Discovery and analysis of inflammatory disease-related genes using cDNA microarrays

(inflammation/human genome analysis/gene discovery)

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ABSTRACT   cDNA microarray technology is used to profile complex diseases and discover novel disease-related genes. In inflammatory disease such as rheumatoid arthritis, expression patterns of diverse cell types contribute to the pathology. We have monitored gene expression in this disease state with a microarray of selected human genes of probable significance in inflammation as well as with genes expressed in peripheral human blood cells. Messenger RNA from cultured macrophages, chondrocyte cell lines, primary chondrocytes, and synoviocytes provided expression profiles for the selected cytokines, chemokines, DNA binding proteins, and matrix-degrading metalloproteinases. Comparisons between tissue samples of rheumatoid arthritis and inflammatory bowel disease verified the involvement of many genes and revealed novel participation of the cytokine interleukin 3, chemokine Groα and the metalloproteinase matrix metallo-elastase in both diseases. From the peripheral blood library, tissue inhibitor of metalloproteinase 1, ferritin light chain, and manganese superoxide dismutase genes were identified as expressed differentially in rheumatoid arthritis compared with inflammatory bowel disease. These results successfully demonstrate the use of the cDNA microarray system as a general approach for dissecting human diseases.

The recently described cDNA microarray or DNA-chip technology allows expression monitoring of hundreds and thousands of genes simultaneously and provides a format for identifying genes as well as changes in their activity (1, 2). Using this technology, two-color fluorescence patterns of differential gene expression in the root versus the shoot tissue of *Arabidopsis* were obtained in a specific array of 48 genes (1). In another study using a 1000 gene array from a human peripheral blood library, novel genes expressed by T cells were identified upon heat shock and protein kinase C activation (3).

The technology uses cDNA sequences or cDNA inserts of a library for PCR amplification that are arrayed on a glass slide with high speed robotics at a density of 1000 cDNA sequences per cm². These microarrays serve as gene targets for hybridization to cDNA probes prepared from RNA samples of cells or tissues. A two-color fluorescence labeling technique is used in the preparation of the cDNA probes such that a simultaneous hybridization but separate detection of signals provides the comparative analysis and the relative abundance of specific genes expressed (1, 2). Microarrays can be constructed from specific cDNA clones of interest, a cDNA library, or a select number of open reading frames from a genome sequencing database to allow a large-scale functional analysis of expressed sequences.

Because of the wide spectrum of genes and endogenous mediators involved, the microarray technology is well suited for analyzing chronic diseases. In rheumatoid arthritis (RA), inflammation of the joint is caused by the gene products of many different cell types present in the synovium and cartilage tissues plus those infiltrating from the circulating blood. The autoimmune and inflammatory nature of the disease is a cumulative result of genetic susceptibility factors and multiple responses, paracrine and autocrine in nature, from macrophages, T cells, plasma cells, neutrophils, synovial fibroblasts, chondrocytes, etc. Growth factors, inflammatory cytokines (4), and the chemokines (5) are the important mediators of this inflammatory process. The ensuing destruction of the cartilage and bone by the invading synovial tissue includes the actions of prostaglandins and leukotrienes (6), and the matrix degrading metalloproteinases (MMPs). The MMPs are an important class of Zn-dependent metallo-endoproteinases that can collectively degrade the proteoglycan and collagen components of the connective tissue matrix (7).

This paper presents a study in which the involvement of select classes of molecules in RA was examined. Also investigated were 1000 human genes randomly selected from a peripheral human blood cell library. Their differential and quantitative expression analysis in cells of the joint tissue, in diseased RA tissue and in inflammatory bowel disease (IBD) tissues was conducted to demonstrate the utility of the microarray method to analyze complex diseases by their pattern of gene expression. Such a survey provides insight not only into the underlying cause of the pathology, but also provides the opportunity to selectively target genes for disease intervention by appropriate drug development and gene therapies.

METHODS

Microarray Design, Development, and Preparation. Two approaches for the fabrication of cDNA microarrays were used in this study. In the first approach, known human genes of probable significance in RA were identified. Regions of the clones, preferably 1 kb in length, were selected by their proximity to the 3' end of the cDNA and for areas of least identity to related and repetitive sequences. Primers were synthesized to amplify the target regions by standard PCR protocols (3). Products were

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verified by gel electrophoresis and purified with Qiaquick 96-well purification kit (Qiagen, Chatsworth, CA), lyophilized (Savant), and resuspended in 5 μl of 3× standard saline citrate (SSC) buffer for arraying. In the second approach, the microarray containing the 1056 human genes from the peripheral blood lymphocyte library was prepared as described (3).

Tissue Specimens. Rheumatoid synovial tissue was obtained from patients with late stage classic RA undergoing remedial synovectomy or arthroplasty of the knee. Synovial tissue was separated from any associated connective tissue or fat. One gram of each synovial specimen was subjected to RNA extraction within 40 min of surgical excision, or explants were cultured in serum-free medium to examine any changes under *in vitro* conditions. For IBD, specimens of macroscopically inflamed lower intestinal mucosa were obtained from patients with Crohn disease undergoing remedial surgery. The hypertrophied mucosal tissue was separated from underlying connective tissue and extracted for RNA.

Cultured Cells. The Mono Mac-6 (MM6) monocytic cells (8) were grown in RPMI medium. Human chondrosarcoma SW1353 cells, primary human chondrocytes, and synoviocytes (9, 10) were cultured in DMEM; all culture media were supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 500 units/ml penicillin. Treatment of cells with lipopolysaccharide (LPS) endotoxin at 30 ng/ml, phorbol 12-myristate 13-acetate (PMA) at 50 ng/ml, tumor necrosis factor α (TNF-α) at 50 ng/ml, interleukin (IL)-1β at 30 ng/ml, or transforming growth factor-β (TGF-β) at 100 ng/ml is described in the figure legends.

Fluorescent Probe, Hybridization, and Scanning. Isolation of mRNA, probe preparation, and quantitation with *Arabidopsis* control mRNAs was essentially as described (3) except for the following minor modification. Following the reverse transcriptase step, the appropriate Cy3- and Cy5-labeled samples were pooled; mRNA degraded by heating the sample to 65°C for 10 min with the addition of 5 μl of 0.5M NaOH plus 0.5 ml of 10 mM EDTA. The pooled cDNA was purified from unincorporated nucleotides by gel filtration in Centri-spin columns (Princeton Separations, Adelphia, NJ). Samples were lyophilized and dissolved in 6 μl of hybridization buffer (5× SSC plus 0.2% SDS). Hybridizations, washes, scanning, quantitation procedures, and pseudocolor representations of fluorescent images have been described (3). Scans for the two fluorescent probes were normalized either to the fluorescence intensity of *Arabidopsis* mRNAs spiked into the labeling reactions (see Figs. 2–4) or to the signal intensity of β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; see Fig. 5).

RESULTS

Ninety-Six-Gene Microarray Design. The actions of cytokines, growth factors, chemokines, transcription factors, MMPs, prostaglandins, and leukotrienes are well recognized in inflammatory disease, particularly RA (11–14). Fig. 1 displays the selected genes for this study and also includes control cDNAs of housekeeping genes such as β-actin and GAPDH and genes from *Arabidopsis* for signal normalization and quantitation (row A, columns 1–12).

Defining Microarray Assay Conditions. Different lengths and concentrations of target DNA were tested by arraying PCR-

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	BLANK	HAT1 / HAT1	HAT1 / HAT1	HAT4 / HAT4	HAT4 / HAT4	HAT22 / HAT22	HAT22 / HAT22	YES23 / YES23	YES23 / YES23	BACTIN / β-actin	G3PDH / G3PDH
B	IL1A / IL-1α	IL1B / IL-1β	IL1RA / IL-1RA	IL2 / IL-2	IL3 / IL-3	IL4 / IL-4	IL6 / IL-6	IL6R / IL-6R	IL7 / IL-7	CFOS / c-fos	CJUN / c-jun	RFRA1 / Rat Fra-1
C	IL8 / IL-8	IL9 / IL-9	IL10 / IL-10	ICE / ICE	IFNG / IFNγ	GCSF / G-CSF	MCSF / M-CSF	GMCSF / GM-CSF	TNFB.1 / TNFβ	CREL / c-rel	NFKB50 / NFκBp50	NFKB65.1 / NFκBp65
D	TNFA.1 / TNFα	TNFA.2 / TNFα	TNFA.3 / TNFα	TNFA.4 / TNFα	TNFA.5 / TNFα	TNFRI.1 / TNFrI	TNFRI.2 / TNFrI	TNFRII.1 / TNFrII	TNFRII.2 / TNFrII	NFKB65.2 / NFκBp65	IKB / IκB	CREB2 / CREB2
E	STR1 / Strom-1	STR2-3' / Strom-2	STR3 / Strom-3	COL1 / Coll-1	COL1-3' / Coll-1.3'	COL2.1 / Coll-2	COL2.2 / Coll-2	COL3 / Coll-3	COX1 / Cox-1	COX2 / Cox-2	12LO / 12-LO	15LO / 15-LO
F	GELA.1 / Gel-A	GELB / Gel-B	HME / Elastase	MTMMP / MT-MMP	PUMP1 / Matrilysin	TIMP1 / TIMP-1	TIMP2 / TIMP-2	TIMP3 / TIMP-3	ICAM1 / ICAM-1	VCAM / VCAM	5LO.1 / 5-LO	CPLA2.2 / cPLA2
G	EGF / EGF	FGFA / FGF acidic	FGFB / FGF basic	IGF1 / IGF-I	IGF2 / IGF-II	TGFA / TGFα	TGFB / TGFβ	PDGFB / PDGFβ	CALCTN / Calcitonin	GH1 / GH-1	GRO / GRO1α	GCR / GCR
H	MCP1.1 / MCP-1	MCP1.1 / MCP-1	MIP1A / MIP-1α	MIP1B / MIP-1β	MIF / MIF	RANTES / RANTES	INOS / iNOS	LDLR / LDLR	ALU.1 / IL-10	ALU.2 / TNFRp70	ALU.3 / IL-10	POLYA / LDLR

□ *A. thaliana* controls
▦ Human controls
⬚ Cytokines and related genes
Transcription factors and related genes
⬚ MMP's and related genes
⬚ Chemokines
▨ Growth factors and related genes
■ Other genes

FIG. 1. Ninety-six-element microarray design. The target element name and the corresponding gene are shown in the layout. Some genes have more than one target element to guarantee specificity of signal. For TNF the targets represent decreasing lengths of 1, 0.8, 0.6, 0.4, and 0.2 kb from left to right.

amplified products ranging from 0.2 to 1.2 kb at concentrations of 1 µg/µl or less. No significant difference in the signal levels was observed within this range of target size and only with 0.2-kb length was a signal reduced upon an 8-fold dilution of the 1 µg/µl sample (data not shown). In this study the average length of the targets was 1 kb, with a few exceptions in the range of ≈300 bp, arrayed at a concentration of 1 µg/µl. Normally one PCR provided sufficient material to fabricate up to 1000 microarray targets. In considering positional effects in the development of the targets for the microarrays, selection was biased toward the 3' proximal regions, because the signal was reduced if the target fragment was biased toward the 5' end (data not shown). This result was anticipated since the hybridizing probe is prepared by reverse transcription with oligo(dT)-primed mRNA and is richer in 3' proximal sequences. Cross-hybridizations of probes to targets of a gene family were analyzed with the matrix metal-

loproteinases as the example because they can show regions of sequence identities of greater than 70%. With collagenase-1 (Col-1) and collagenase-2 (Col-2) genes as targets with up to 70% sequence identity, and stromelysin-1 (Strom-1) and stromelysin-2 (Strom-2) genes with different degrees of identity, our results showed that a short region of overlap, even with 70–90% sequence identity, produced a low level of cross-hybridization. However, shorter regions of identity spread over the length of the target resulted in cross-hybridization (data not shown). For closely related genes, targets were designed by avoiding long stretches of homology. For members of a gene family two or more target regions were included to discriminate between specificity of signal versus cross-hybridization.

Monitoring Differential Expression in Cultured Cell Lines. In RA tissue, the monocyte/macrophage population plays a prominent role in phagocytic and immunomodulatory activities. Typ-
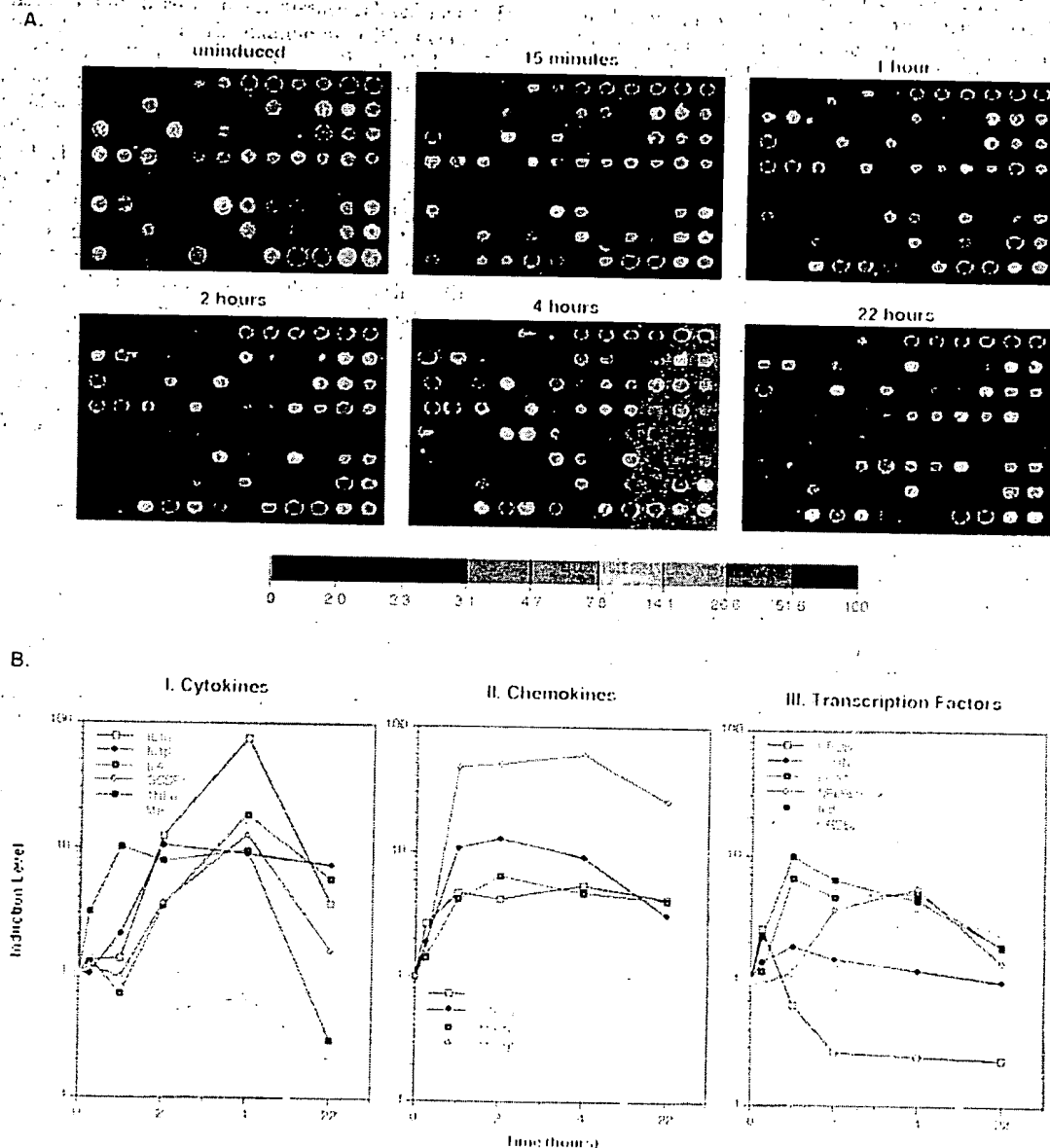
FIG. 2.   Time course for LPS/PMA-induced MM6 cells. Array elements are described in Fig. 1. (*A*) Pseudocolor representations of fluorescent scans correspond to gene expression levels at each time point. The array is made up of 8 *Arabidopsis* control targets and 86 human cDNA targets, the majority of which are genes with known or suspected involvement in inflammation. The color bars provide a comparative calibration scale between arrays and are derived from the *Arabidopsis* mRNA samples that are introduced in equal amounts during probe preparation. Fluorescent probes were made by labeling mRNA from untreated MM6 cells or LPS and PMA treated cells. mRNA was isolated at indicated times after induction. (*B I–III*) The two-color samples were cohybridized, and microarray scans provided the data for the levels of select transcripts at different time points relative to abundance at time zero. The analysis was performed using normalized data collected from 8-bit images.

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ically these cells, when triggered by an immunogen, produce the proinflammatroy cytokines TNF and IL-1. We have used the monocyte cell line MM6 and monitored changes in gene expression upon activation with LPS endotoxin, a component of Gram-negative bacterial membranes, and PMA, which augments the action of LPS on TNF production (15). RNA was isolated at different times after induction and used for cDNA probe preparation. From this time course it was clear that TNF expression was induced within 15 min of treatment, reached maximum levels in 1 hr, remained high until 4 hr and subsequently declined (Fig. 2A). Many other cytokine genes were also transiently activated, such as IL-1α and -β, IL-6, and granulocyte colony-stimulating factor (GCSF). Prominent chemokines activated were IL-8, macrophage inflammatory protein (MIP)-1β, more so than MIP-1α, and Groα or melanoma growth stimulatory factor. Migration inhibitory factor (MIF) expressed in the uninduced state declined in LPS-activated cells. Of the immediate early genes, the noticeable ones were c-fos, fra-1, c-jun, NF-κBp50, and IκB, with c-rel expression observed even in the uninduced state (Fig. 2B). These expression patterns are consistent with reported patterns of activation of certain LPS- and PMA-induced genes (12). Demonstrated here is the unique ability of this system to allow parallel visualization of a large number of gene activities over a period of time.

SW1353 cells is a line derived from malignant tumors of the cartilage and behaves much like the chondrocytes upon stimulation with TNF and IL-1 in the expression of MMPs (9). In addition to confirming our earlier observations with Northern blots on Strom-1, Col-1, and Col-3 expression (9), gelatinase (Gel) A, putative metalloproteinase (PUMP)-1 membrane-

type matrix metalloproteinase, tissue inhibitors of matrix metalloproteinases or tissue inhibitor of metalloproteinase 1 (TIMP-1), -2, and -3 were also expressed by these cells together with the human matrix metallo-elastase (HME; Fig. 3A). HME induction was estimated to be ~50-fold and was greater than any of the other MMPs examined (Fig. 3B). This result was unexpected because HME is reportedly expressed only by alveolar macrophage and placental cells (16). Expression of the cytokines and chemokines, IL-6, IL-8, MIF, and MIP-1β was also noted. A variety of other genes, including certain transcription factors, were also up-regulated (Fig. 3), but the overall time-dependent expression of genes in the SW1353 cells was qualitatively distinct from the MM6 cells.

Quantitation of differential gene expression (Figs. 2B and 3B) was achieved with the simultaneous hybridization of Cy3-labeled cDNA from untreated cells and Cy5-labeled cDNA from treated samples. The estimated increases in expression from these microarrays for a select number of genes including IL-1β, IL-8, MIP-1β, TNF, HME, Col-1, Col-3, Strom-1, and Strom-2 were compared with data collected from dot blot analysis. Results (not shown) were in close agreement and confirmed our earlier observations on the use of the microarray method for the quantitation of gene expression (3).

Expression Profiles in Primary Chondrocytes and Synoviocytes of Human RA Tissue. Given the sensitivity and the specificity of this method, expression profiles of primary synoviocytes and chondrocytes from diseased tissue were examined. Without prior exposure to inducing agents, low level expression of c-jun, GCSF, IL-3, TNF-β, MIF, and RANTES (regulated upon activation, normal T cell expressed and secreted) was seen as well as expression of MMPs, GelA, Strom-1, Col-1, and the three TIMPs. In this case, Col-2 hybridization was considered to be nonspecific because the second Col-2 target taken from the 3' end of the gene gave no
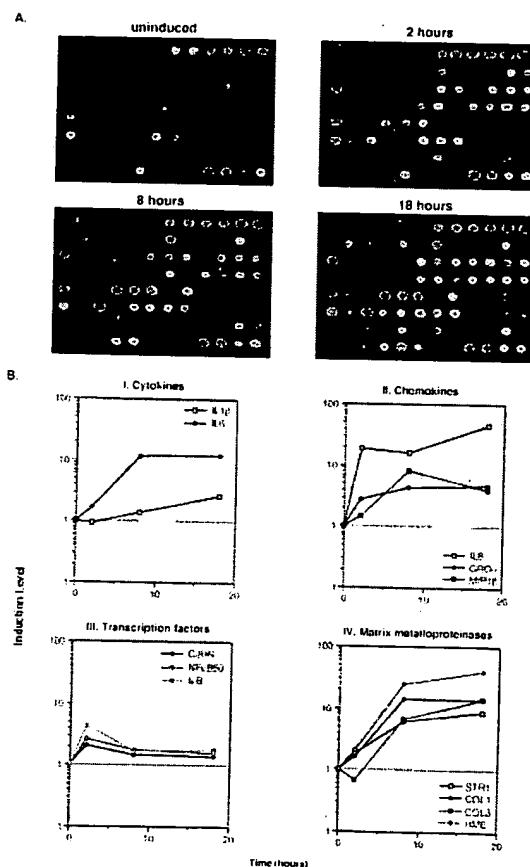
FIG. 3. Time course for IL-1β and TNF-induced SW1353 cells using the inflammation array (Fig. 1). (*A*) Pseudocolor representation of fluorescent scans correspond to gene expression levels at each time point. (*B I–IV*) Relative levels of selected genes at different time points compared with time zero.
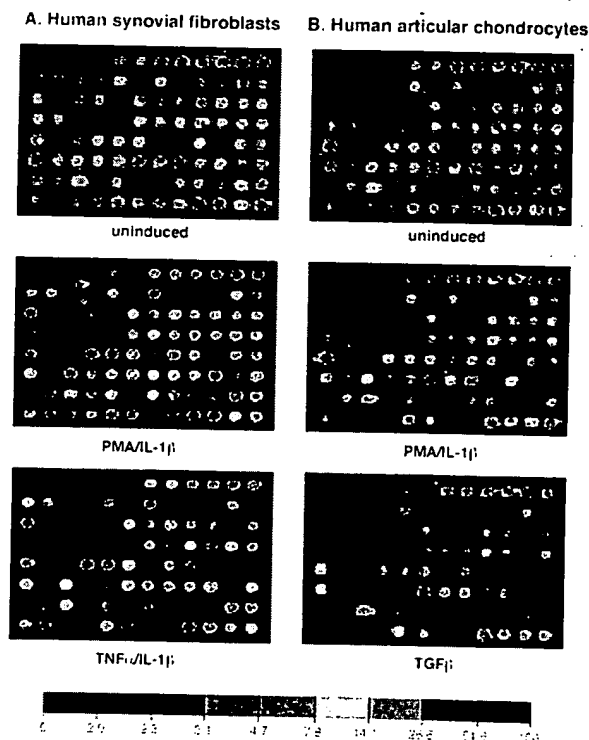
FIG. 4. Expression profiles for early passage primary synoviocytes and chondrocytes isolated from RA tissue, cultured in the presence of 10% fetal calf serum and activated with PMA and IL-1β, or TNF and IL-1β, or TGF-β for 18 hr. The color bars provide a comparative calibration scale between arrays and are derived from the *Arabidopsis* mRNA samples that are introduced in equal amounts during probe preparation

signal. Treatment more so with PMA and IL-1, than TNF and IL-1, produced a dramatic up-regulation in expression of several genes in both of these primary cell types. These genes are as follows: the cytokine IL-6, the chemokines IL-8 and Gro-1α, and the MMPs; Strom-1, Col-1, Col-3, and HME; and the adhesion molecule, vascular cell adhesion molecule 1 (VCAM-1). The surprise again is HME expression in these primary cells, for reasons discussed above. From these results, the expression profiles of synoviocytes and the chondrocytes appear very similar; the differences are more quantitative than qualitative. Treatment of the primary chondrocytes with the anabolic growth factor TGF-β had an interesting profile in that it produced a remarkable down-regulation of genes expressed in both the untreated and induced state (Fig. 4).

Given the demonstrated effectiveness of this technology, a comparative analysis of two different inflammatory disease states was conducted with probes made from RA tissue and IBD samples. RA samples were from late stage rheumatoid synovial tissue, and IBD specimens were obtained from inflamed lower intestinal mucosa of patients with Crohn disease. With both the 96-element known gene microarray and the 1000-gene microarray of cDNAs selected from a peripheral human blood cell library (3), distinct differences in gene expression patterns were evident. On the 96-gene array, RA tissue samples from different affected individuals gave similar profiles (data not shown) as did different samples from the same individual (Fig. 5). These patterns were notably similar to those observed with primary synoviocytes and chondrocytes (Fig. 4). Included in the list of prominently up-regulated genes are IL-6, the MMPs Strom-1, Col-1, GelA, HME, and in

certain samples PUMP, TIMPs, particularly TIMP-1 and TIMP-3, and the adhesion molecule VCAM. Discernible levels of macrophage chemotactic protein 1 (MCP-1), MIF and RANTES were also noted. IBD samples were in comparison, rather subdued although IL-1 converting enzyme (ICE), TIMP-1, and MIF were notable in all the three different IBD samples examined here. In IBD-A, one of three individual samples, ICE, VCAM, Groα, and MMP expression was more pronounced than in the others.

We also made use of a peripheral blood cDNA library (3) to identify genes expressed by lymphocytes infiltrating the inflamed tissues from the circulating blood. With the 1046-element array of randomly selected cDNAs from this library, probes made from RA and IBD samples showed hybridizations to a large number of genes. Of these, many were common between the two disease tissues while others were differentially expressed (data not shown). A complete survey of these genes was beyond the scope of this study, but for this report we picked three genes that were up-regulated in the RA tissue relative to IBD. These cDNAs were sequenced and identified by comparison to the GenBank database. They are TIMP-1, apoferritin light chain, and manganese superoxide dismutase (MnSOD). Differential expression of MnSOD was only observed in samples of RA tissue explants maintained in growth medium without serum for anywhere between 2 to 16 hr. These results also indicate that the expression profile of genes can be altered when explants are transferred to culture conditions.

DISCUSSION

The speed, ease, and feasibility of simultaneously monitoring differential expression of hundreds of genes with the cDNA microarray based system (1–3) is demonstrated here in the analysis of a complex disease such as RA. Many different cell types in the RA tissue; macrophages, lymphocytes, plasma cells, neutrophils, synoviocytes, chondrocytes, etc. are known to contribute to the development of the disease with the expression of gene products known to be proinflammatory. They include the cytokines, chemokines, growth factors, MMPs, eicosanoids, and others (7, 11–14), and the design of the 96-element known gene microarray was based on this knowledge and depended on the availability of the genes. The technology was validated by confirming earlier observations on the expression of TNF by the monocyte cell line MM6, and of Col-1 and Col-3 expression in the chondrosarcoma cells and articular chondrocytes (9, 12). In our time-dependent survey the chronological order of gene activities in and between gene families was compared and the results have provided unprecedented profiles of the cytokines (TNF, IL-1, IL-6, GCSF, and MIF), chemokines (MIP-1α, MIP-1β, IL-8, and Gro-1), certain transcription factors, and the matrix metalloproteinases (GelA, Strom-1, Col-1, Col-3, HME) in the macrophage cell line MM6 and in the SW1353 chondrosarcoma cells.

Earlier reports of cytokine production in the diseased state had established a model in which TNF is a major participant in RA. Its expression reportedly preceded that of the other cytokines and effector molecules (4). Our results strongly support these results as demonstrated in the time course of the MM6 cells where TNF induction preceded that of IL-1α and IL-β followed by IL-6 and GCSF. These expression profiles demonstrate the utility of the microarrays in determining the hierarachy of signaling events.

In the SW1353 chondrosarcoma cells, all the known MMPs and TIMPs were examined simultaneously. HME expression was discovered, which previously had been observed in only the stromal cells and alveolar macrophages of smoker's lungs and in placental tissue. Its presence in cells of the RA tissue is meaningful because its activity can cause significant destruction of elastin and basement membrane components (16, 17). Expression profiles of synovial fibroblasts and articular chondrocytes were remarkably similar and not too different from the SW1353 cells, indicating that the fibroblast and the chondrocyte can play equally aggressive roles in joint erosion. Prominent genes expressed were
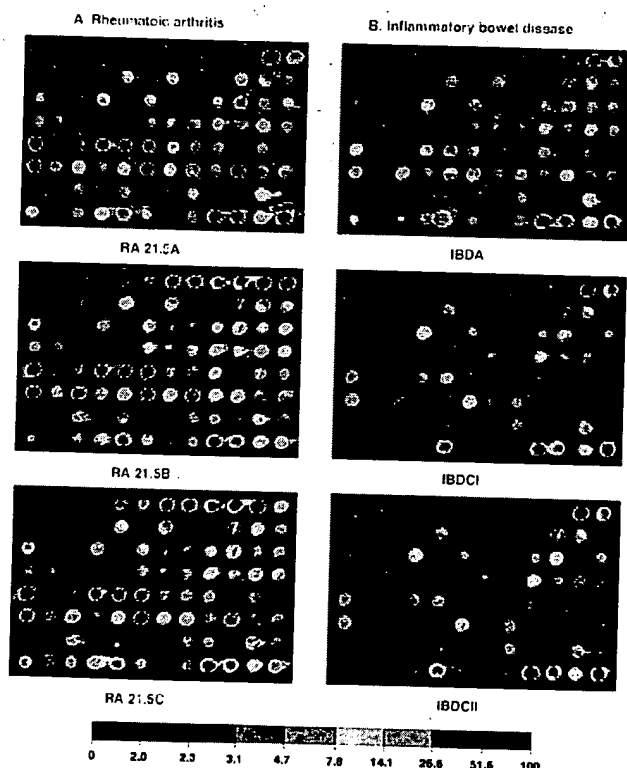
FIG. 5. Expression profiles of RA tissue (*A*) and IBD tissue (*B*). mRNA from RA tissue samples obtained from the same individual was isolated directly after excision (RA 21.5A) or maintained in culture without serum for 2 hr (RA 21.5B) or for 6 hr (RA 21.5C). Profiles from tissue samples of two other individuals (data not shown) were remarkably similar to the ones shown here. IBD-A and IBD-CI are from mRNA samples prepared directly after surgery from two separate individuals. For the IBD-CII probe, the tissue sample was cultured in medium without serum for 2 hr before mRNA preparation.

the MMPs, but chemokines and cytokines were also produced by these cells. The effect of the anabolic growth factor TGF-β was profoundly evident in demonstrating the down regulation of these catabolic activities.

RA tissue samples undeniably reflected profiles similar to the cell types examined. Active genes observed were IL-3, IL-6, ICE, the MMPs including HME and TIMPs, chemokines IL-8, Groα, MIP, MIF, and RANTES, and the adhesion molecule VCAM. Of the growth factors, fibroblast growth factor β was observed most frequently. In comparison, the expression patterns in the other inflammatory state (i.e., IBD) were not as marked as in the RA samples, at least as obtained from the tissue samples selected for this study.

As an alternative approach, the 1046 cDNA microarray of randomly selected genes from a lymphocyte library was used to identify genes expressed in RA tissue (3). Many genes on this array hybridized with probes made from both RA and IBD tissue samples. The results are not surprising because inflammatory tissue is abundantly supplied with cell types infiltrating from the circulating blood, made apparent also by the high levels of chemokine expression in RA tissue. Because of the magnitude of the effort required to identify all the hybridized genes, we have for this report chosen to describe only three differentially expressed genes mainly to verify this method of analysis.

Of the large number of genes observed here, a fair number were already known as active participants in inflammatory disease. These are TNF, IL-1, IL-6, IL-8, GCSF, RANTES, and VCAM. The novel participants not previously reported are HME, IL-3, ICE, and Groα. With our discovery of HME expression in RA, this gene becomes a target for drug intervention. ICE is a cysteine protease well known for its IL-1β processing activity (18), and recognized for its role in apoptotic cell death (19). Its expression in RA tissue is intriguing. IL-3 is recognized for its growth-promoting activity in hematopoietic cell lineages, is a product of activated T cells (20), and its expression in synoviocytes and chondrocytes of RA tissue is a novel observation.

Like IL-8, Groα, is a C-X-C subgroup chemokine and is a potent neutrophil and basophil chemoattractant. It down-regulates the expression of types I and III interstitial collagens (21, 22) and is seen here produced by the MM6 cells, in primary synoviocytes, and in RA tissue. With the presence of RANTES, MCP, and MIP-1β, the C-C chemokines (23) migration and infiltration of monocytes, particularly T cells, into the tissue is also enhanced (5) and aid in the trafficking and recruitment of leukocytes into the RA tissue. Their activation, phagocytosis, degranulation, and respiratory bursts could be responsible for the induction of MnSOD in RA. MnSOD is also induced by TNF and IL-1 and serves a protective function against oxidative damage. The induction of the ferritin light chain encoding gene in this tissue may be for reasons similar to those for MnSOD. Ferritin is the major intracellular iron storage protein and it is responsive to intracellular oxidative stress and reactive oxygen intermediates generated during inflammation (24, 25). The active expression of TIMP-1 in RA tissue, as detected by the 1000-element array, is no surprise because our results have repeatedly shown TIMP-1 to be expressed in the constitutive and induced states of RA cells and tissues.

The suitability of the cDNA microarray technology for profiling diseases and for identifying disease related genes is well documented here. This technology could provide new targets for drug development and disease therapies, and in doing so allow for improved treatment of chronic diseases that are challenging because of their complexity.

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REPORTS

Axl1p sequence following Ser[209] and occurs within the domain of Axl1p that shows homology with hIDE (14). To delete the complete STE23 sequence and create the ste23Δ::URA3 mutation, polymerase chain reaction (PCR) primers (5'-TCGGAAGACCTCAT-TCTTGCTCATTTTGATATTGCTC- TGTAGATTG-TACTGAGAGTGCAC-3'; and 5'-GCTACAAACAGC-GTCGACTTGAATGCCCCGACATCTTCGACTGT-GCGGTATTTCACACCG-3') were used to amplify the URA3 sequence of pRS316, and the reaction product was transformed into yeast for one-step gene replacement [R. Rothstein, Methods Enzymol. 194, 281 (1991)]. To create the axl1Δ::LEU2 mutation contained on p114, a 5.0-kb Sal I fragment from pAXL1 was cloned into pUC19, and an internal 4.0-kb Hpa I–Xho I fragment was replaced with a LEU2 fragment. To construct the ste23Δ::LEU2 allele (a deletion corresponding to 931 amino acids) carried on p153, a LEU2 fragment was used to replace the 2.8-kb Pml I–Ed136 I fragment of STE23, which occurs within a 6.2-kb Hind III–Bgl II genomic fragment carried on pSP72 (Promega). To create YEpMFA1, a 1.6-kb Bam HI fragment containing MFA1, from pKK16 [K. Kuchler, R. E. Sterne, J. Thorner, EMBO J. 8, 3973 (1989)], was ligated into the Bam HI site of YEp351 [J. E. Hill, A. M. Myers, T. J. Koerner, A. Tzagoloff, Yeast 2, 163 (1986)].

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29. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
30. A W303 1A derivative, SY2625 (MATa ura3-1 leu2-3, 112 trp1-1 ade2-1 can1-100 ssi1Δ mfa2Δ::FUS1-lacZ his3Δ::FUS1-HIS3), was the parent strain for the mutant search. SY2625 derivatives for the mating assays, secreted pheromone assays, and the pulse-chase experiments included the following strains: Y49 (ste22-1), Y115 (mfa1Δ::LEU2), Y142 (axl1::URA3), Y173 (axl1Δ::LEU2), Y220 (axl1::URA3 ste23Δ::URA3), Y221 (ste23Δ::URA3), Y231 (axl1Δ::LEU2 ste23Δ::LEU2), and Y233 (ste23Δ::LEU2). MATα derivatives of SY2625 included the following strains: Y199 (SY2625 made MATα), Y278 (ste22-1), Y195 (mfa1Δ::LEU2), Y196 (axl1Δ::LEU2), and Y197 (axl1::URA3). The EG123 (MATa leu2 ura3 trp1 can1 his4) genetic background was used to create a set of strains for analysis of bud site selection. EG123 derivatives included the following strains: Y175 (axl1Δ::LEU2), Y223 (axl1::URA3), Y234 (ste23Δ::LEU2), and Y272 (axl1Δ::LEU2 ste23Δ::LEU2). MATα derivatives of EG123 included the following strains: Y214 (EG123 made MATα) and Y293 (axl1Δ::LEU2). All strains were generated by means of standard genetic or molecular methods involving the appropriate constructs (23). In particular, the axl1 ste23 double mutant strains were created by crossing of the appropriate MATa ste23 and MATα axl1 mutants, followed by sporulation of the resultant diploid and isolation of the double mutant from nonparental di-type tetrads. Gene disruptions were confirmed with either PCR or Southern (DNA) analysis.
31. p129 is a YEp352 [J. E. Hill, A. M. Myers, T. J. Koerner, A. Tzagoloff, Yeast 2, 163 (1986)] plasmid containing a 5.5-kb Sal I fragment of pAXL1. p151 was derived from p129 by insertion of a linker at the Bgl II site within AXL1, which led to an in-frame insertion of the hemagglutinin (HA) epitope (DGYPYDVPDYA) (29) between amino acids 854 and 855 of the AXL1 prod-

uct. pC225 is a KS+ (Stratagene) plasmid containing a 0.5-kb Bam HI–Sst I fragment from pAXL1. Substitution mutations of the proposed active site of Axl1p were created with the use of pC225 and site-specific mutagenesis involving appropriate synthetic oligonucleotides (axl1-H68A, 5'-GTGCTCACAAAGCGCT-GCCAAACCGGC-3'; axl1-E71A, 5'-AAGAATCAT-GTGCGCACAAAGGTGCGC-3'; and axl1-E71D, 5'-AAGAATCATGTGATCACAAAGGTGCGC-3'). The mutations were confirmed by sequence analysis. After mutagenesis, the 0.4-kb Bam HI–Msc I fragment from the mutagenized pC225 plasmids was transferred into pAXL1 to create a set of pRS316 plasmids carrying different AXL1 alleles, p124 (axl1-H68A), p130 (axl1-E71A), and p132 (axl1-E71D). Similarly, a set of HA-tagged alleles carried on YEp352 were created after replacement of the p151 Bam HI–Msc I fragment, to generate p161 (axl1-E71A), p162 (axl1-

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Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA Microarray

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A high-capacity system was developed to monitor the expression of many genes in parallel. Microarrays prepared by high-speed robotic printing of complementary DNAs on glass were used for quantitative expression measurements of the corresponding genes. Because of the small format and high density of the arrays, hybridization volumes of 2 microliters could be used that enabled detection of rare transcripts in probe mixtures derived from 2 micrograms of total cellular messenger RNA. Differential expression measurements of 45 Arabidopsis genes were made by means of simultaneous, two-color fluorescence hybridization.

The temporal, developmental, topographical, histological, and physiological patterns in which a gene is expressed provide clues to its biological role. The large and expanding database of complementary DNA (cDNA) sequences from many organisms (1) presents the opportunity of defining these patterns at the level of the whole genome.

For these studies, we used the small flowering plant Arabidopsis thaliana as a model organism. Arabidopsis possesses many advantages for gene expression analysis, including the fact that it has the smallest genome of any higher eukaryote examined to date (2). Forty-five cloned Arabidopsis cDNAs (Table 1), including 14 complete sequences and 31 expressed sequence tags (ESTs), were used as gene-specific targets. We obtained the ESTs by selecting cDNA clones at random from an Arabidopsis cDNA library. Sequence analysis revealed that 28 of the 31 ESTs matched sequences

in the database (Table 1). Three additional cDNAs from other organisms served as controls in the experiments.

The 48 cDNAs, averaging ~1.0 kb, were amplified with the polymerase chain reaction (PCR) and deposited into individual wells of a 96-well microtiter plate. Each sample was duplicated in two adjacent wells to allow the reproducibility of the arraying and hybridization process to be tested. Samples from the microtiter plate were printed onto glass microscope slides in an area measuring 3.5 mm by 5.5 mm with the use of a high-speed arraying machine (3). The arrays were processed by chemical and heat treatment to attach the DNA sequences to the glass surface and denature them (3). Three arrays, printed in a single lot, were used for the experiments here. A single microtiter plate of PCR products provides sufficient material to print at least 500 arrays.

Fluorescent probes were prepared from total Arabidopsis mRNA (4) by a single round of reverse transcription (5). The Arabidopsis mRNA was supplemented with human acetylcholine receptor (AChR) mRNA at a dilution of 1:10,000 (w/w) before cDNA synthesis, to provide an internal standard for calibration (5). The resulting fluorescently labeled cDNA mixture was hybridized to an array at high stringency (6) and scanned

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with a laser (3). A high-sensitivity scan gave signals that saturated the detector at nearly all of the Arabidopsis target sites (Fig. 1A). Calibration relative to the AChR mRNA standard (Fig. 1A) established a sensitivity limit of ~1:50,000. No detectable hybridization was observed to either the rat glucocorticoid receptor (Fig. 1A) or the yeast TRP4 (Fig. 1A) targets even at the highest scanning sensitivity. A moderate-sensitivity scan of the same array allowed linear detection of the more abundant transcripts (Fig. 1B). Quantitation of both scans revealed a range of expression levels spanning three orders of magnitude for the 45 genes tested (Table 2). RNA blots (7) for several genes (Fig. 2) corroborated the expression levels measured with the microarray to within a factor of 5 (Table 2).

Differential gene expression was investigated with a simultaneous, two-color hybridization scheme, which served to minimize experimental variation inherent in the comparison of independent hybridizations. Fluorescent probes were prepared from two mRNA sources with the use of reverse transcriptase in the presence of fluorescein- and lissamine-labeled nucleotide analogs, respectively (5). The two probes were then mixed together in equal proportions, hybridized to a single array, and scanned separately for fluorescein and lissamine emission after independent excitation of the two fluorophores (3).

To test whether overexpression of a single gene could be detected in a pool of total Arabidopsis mRNA, we used a microarray to analyze a transgenic line overexpressing the single transcription factor HAT4 (8). Fluorescent probes representing mRNA from wild-type and HAT4-transgenic plants were labeled with fluorescein and lissamine, respectively; the two probes were then mixed and hybridized to a single array. An intense hybridization signal was observed at the position of the HAT4 cDNA in the lissamine-specific scan (Fig. 1D), but not in the fluorescein-specific scan of the same array (Fig. 1C). Calibration with AChR mRNA added to the fluorescein and lissamine cDNA synthesis reactions at dilutions of 1:10,000 (Fig. 1C) and 1:100 (Fig. 1D), respectively, revealed a 50-fold elevation of HAT4 mRNA in the transgenic line relative to its abundance in wild-type plants (Table 2). This magnitude of HAT4 overexpression matched that inferred from the Northern (RNA) analysis within a factor of 2 (Fig. 2 and Table 2). Expression of all the other genes monitored on the array differed by less than a factor of 5 between HAT4-transgenic and wild-type plants (Fig 1, C
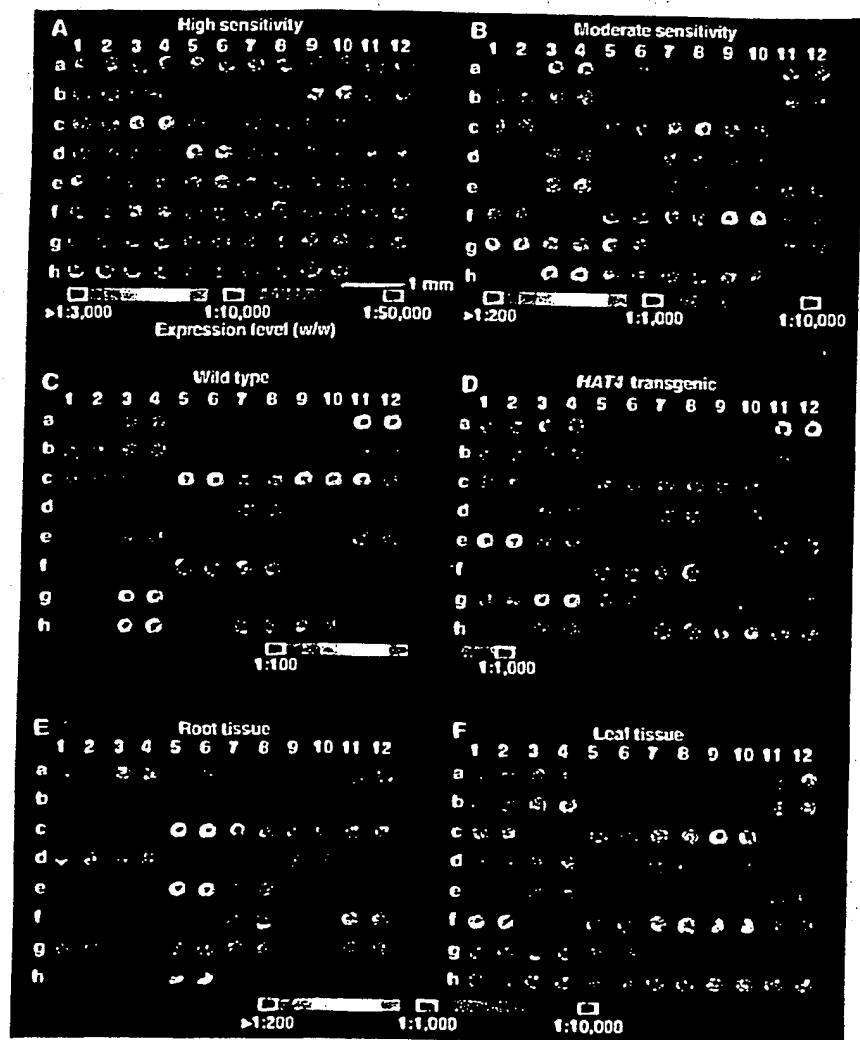
Fig. 1. Gene expression monitored with the use of cDNA microarrays. Fluorescent scans represented in pseudocolor correspond to hybridization intensities. Color bars were calibrated from the signal obtained with the use of known concentrations of human AChR mRNA in independent experiments. Numbers and letters on the axes mark the position of each cDNA. (A) High-sensitivity fluorescein scan after hybridization with fluorescein-labeled cDNA derived from wild-type plants. (B) Same array as in (A) but scanned at moderate sensitivity. (C and D) A single array was probed with a 1:1 mixture of fluorescein-labeled cDNA from wild-type plants and lissamine-labeled cDNA from HAT4-transgenic plants. The single array was then scanned successively to detect the fluorescein fluorescence corresponding to mRNA from wild-type plants (C) and the lissamine fluorescence corresponding to mRNA from HAT4-transgenic plants (D). (E and F) A single array was probed with a 1:1 mixture of fluorescein-labeled cDNA from root tissue and lissamine-labeled cDNA from leaf tissue. The single array was then scanned successively to detect the fluorescein fluorescence corresponding to mRNAs expressed in roots (E) and the lissamine fluorescence corresponding to mRNAs expressed in leaves (F).
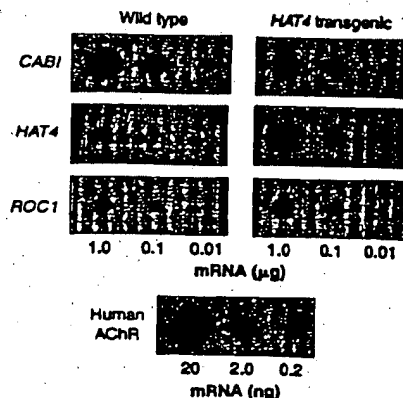
Fig. 2. Gene expression monitored with RNA (Northern) blot analysis. Designated amounts of mRNA from wild-type and HAT4-transgenic plants were spotted onto nylon membranes and probed with the cDNAs indicated. Purified human AChR mRNA was used for calibration.

and D, and Table 2). Hybridization of fluorescein-labeled glucocorticoid receptor cDNA (Fig. 1C) and lissamine-labeled TRP4 cDNA (Fig. 1D) verified the presence of the negative control targets and the lack of optical cross talk between the two fluorophores.

To explore a more complex alteration in expression patterns, we performed a second two-color hybridization experiment with fluorescein- and lissamine-labeled probes prepared from root and leaf mRNA, respectively. The scanning sensitivities for the two fluorophores were normalized by matching the signals resulting from AChR

mRNA, which was added to both cDNA synthesis reactions at a dilution of 1:1000 (Fig. 1, E and F). A comparison of the scans revealed widespread differences in gene expression between root and leaf tissue (Fig. 1, E and F). The mRNA from the light-regulated CABI gene was ~500-fold more abundant in leaf (Fig. 1F) than in root tissue (Fig. 1E). The expression of 26 other genes differed between root and leaf tissue by more than a factor of 5 (Fig. 1, E and F).

The HAT4-transgenic line we examined has elongated hypocotyls, early flowering, poor germination, and altered pigmentation (8). Although changes in expression were

observed for HAT4, large changes in expression were not observed for any of the other 44 genes we examined. This was somewhat surprising, particularly because comparative analysis of leaf and root tissue identified 27 differentially expressed genes. Analysis of an expanded set of genes may be required to identify genes whose expression changes upon HAT4 overexpression; alternatively, a comparison of mRNA populations from specific tissues of wild-type and HAT4-transgenic plants may allow identification of downstream genes.

At the current density of robotic printing, it is feasible to scale up the fabrication process to produce arrays containing 20,000 cDNA targets. At this density, a single array would be sufficient to provide gene-specific targets encompassing nearly the entire repertoire of expressed genes in the Arabidopsis genome (2). The availability of 20,274 ESTs from Arabidopsis (1, 9) would provide a rich source of templates for such studies.

The estimated 100,000 genes in the human genome (10) exceeds the number of Arabidopsis genes by a factor of 5 (2). This modest increase in complexity suggests that similar cDNA microarrays, prepared from the rapidly growing repertoire of human ESTs (1), could be used to determine the expression patterns of tens of thousands of human genes in diverse cell types. Coupling an amplification strategy to the reverse transcription reaction (11) could make it feasible to monitor expression even in minute tissue samples. A wide variety of acute and chronic physiological and pathological conditions might lead to characteristic changes in the patterns of gene expression in peripheral blood cells or other easily sampled tissues. In concert with cDNA microarrays for monitoring complex expression patterns, these tissues might therefore serve as sensitive in vivo sensors for clinical diagnosis. Microarrays of cDNAs could thus provide a useful link between human gene sequences and clinical medicine.

Table 1. Sequences contained on the cDNA microarray. Shown is the position, the known or putative function, and the accession number of each cDNA in the microarray (Fig. 1). All but three of the ESTs used in this study matched a sequence in the database. NADH, reduced form of nicotinamide adenine dinucleotide; ATPase, adenosine triphosphatase; GTP, guanosine triphosphate.

Position	cDNA	Function	Accession number
a1, 2	AChR	Human AChR	·
a3, 4	EST3	Actin	
a5, 6	EST6	NADH dehydrogenase	H36236
a7, 8	AAC1	Actin 1	Z27010
a9, 10	EST12	Unknown	M20016
a11, 12	EST13	Actin	U36594†
b1, 2	CABI	Chlorophyll a/b binding	T45783
b3, 4	EST17	Phosphoglycerate kinase	M85150
b5, 6	GA4	Gibberellic acid biosynthesis	T44490
b7, 8	EST19	Unknown	L37126
b9, 10	GBF-1	G-box binding factor 1	U36595†
b11, 12	EST23	Elongation factor	X63894
c1, 2	EST29	Aldolase	X52256
c3, 4	GBF-2	G-box binding factor 2	T04477
c5, 6	EST34	Chloroplast protease	X63895
c7, 8	EST35	Unknown	R87034
c9, 10	EST41	Catalase	T14152
c11, 12	rGR	Rat glucocorticoid receptor	T22720
d1, 2	EST42	Unknown	M14053
d3, 4	EST45	ATPase	U36596†
d5, 6	HAT1	Homeobox-leucine zipper 1	J04185
d7, 8	EST46	Light harvesting complex	U09332
d9, 10	EST49	Unknown	T04063
d11, 12	HAT2	Homeobox-leucine zipper 2	T76267
e1, 2	HAT4	Homeobox-leucine zipper 4	U09335
e3, 4	EST50	Phosphoribulokinase	M90394
e5, 6	HAT5	Homeobox-leucine zipper 5	T04344
e7, 8	EST51	Unknown	M90416
e9, 10	HAT22	Homeobox-leucine zipper 22	Z33675
e11, 12	EST52	Oxygen evolving	U09336
f1, 2	EST59	Unknown	T21749
f3, 4	KNAT1	Knotted-like homeobox 1	Z34607
f5, 6	EST60	RuBisCO small subunit	U14174
f7, 8	EST69	Translation elongation factor	X14564
f9, 10	PPH1	Protein phosphatase 1	T42799
f11, 12	EST70	Unknown	U34803
g1, 2	EST75	Chloroplast protease	T44621
g3, 4	EST78	Unknown	T43698
g5, 6	ROC1	Cyclophilin	R65481
g7, 8	EST82	GTP binding	L14844
g9, 10	EST83	Unknown	X59152
g11, 12	EST84	Unknown	Z33795
h1, 2	EST91	Unknown	T45278
h3, 4	EST96	Unknown	T13832
h5, 6	SAR1	Synaptobrevin	R64816
h7, 8	EST100	Light harvesting complex	M90418
h9, 10	EST103	Light harvesting complex	Z18205
h11, 12	TRP4	Yeast tryptophan biosynthesis	X03909
			X04273

'Proprietary sequence of Stratagene (La Jolla, California). †No match in the database; novel EST.

Table 2. Gene expression monitoring by microarray and RNA blot analyses; tg, HAT4-transgenic. See Table 1 for additional gene information. Expression levels (w/w) were calibrated with the use of known amounts of human AChR mRNA. Values for the microarray were determined from microarray scans (Fig. 1); values for the RNA blot were determined from RNA blots (Fig. 2).

Gene	Expression level (w/w)	
	Microarray	RNA blot
CABI	1:48	1:83
CABI (tg)	1:120	1:150
HAT4	1:8300	1:6300
HAT4 (tg)	1:150	1:210
ROC1	1:1200	1:1800
ROC1 (tg)	1:260	1:1300

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3. D. Shalon, thesis, Stanford University (1995); _____ and P. O. Brown, in preparation. Microarrays were fabricated on poly-L-lysine–coated microscope slides (Sigma) with a custom-built arraying machine fitted with one printing tip. The tip loaded 1 μl of PCR product (0.5 mg/ml) from 96-well microtiter plates and deposited ~0.005 μl per slide on 40 slides at a spacing of 500 μm. The printed slides were rehydrated for 2 hours in a humid chamber, snap-dried at 100°C for 1 min, rinsed in 0.1% SDS, and treated with 0.05% succinic anhydride prepared in buffer consisting of 50% 1-methyl-2-pyrrolidinone and 50% boric acid. The cDNA on the slides was denatured in distilled water for 2 min at 90°C immediately before use. Microarrays were scanned with a laser fluorescent scanner that contained a computer-controlled XY stage and a microscope objective. A mixed gas, multiline laser allowed sequential excitation of the two fluorophores. Emitted light was split according to wavelength and detected with two photomultiplier tubes. Signals were read into a PC with the use of a 12-bit analog-to-digital board. Additional details of microarray fabrication and use may be obtained by means of e-mail (pbrown@cmgm. stanford.edu).

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5. Polyadenylated [poly(A)⁺] mRNA was prepared from total RNA with the use of Oligotex-dT resin (Qiagen). Reverse transcription (RT) reactions were carried out with a StrataScript RT-PCR kit (Stratagene) modified as follows: 50-μl reactions contained 0.1 μg/μl of Arabidopsis mRNA, 0.1 ng/μl of human AChR mRNA, 0.05 μg/μl of oligo(dT) (21-mer), 1× first strand buffer, 0.03 U/μl of ribonuclease block, 500 μM deoxyadenosine triphosphate (dATP), 500 μM deoxyguanosine triphosphate, 500 μM dTTP, 40 μM deoxycytosine triphosphate (dCTP), 40 μM fluorescein-12-dCTP (or lissamine-5-dCTP), and 0.03 U/μl of StrataScript reverse transcriptase. Reactions were incubated for 60 min at 37°C, precipitated with ethanol, and resuspended in 10 μl of TE (10 mM tris-HCl and 1 mM EDTA, pH 8.0). Samples were then heated for 3 min at 94°C and chilled on ice. The RNA was degraded by adding 0.25 μl of 10 N NaOH followed by a 10-min incubation at 37°C. The samples were neutralized by addition of 2.5 μl of 1 M tris-Cl (pH 8.0) and 0.25 μl of 10 N HCl and precipitated with ethanol. Pellets were washed with 70% ethanol, dried to completion in a speedvac, resuspended in 10 μl of H₂O, and reduced to 3.0 μl in a speedvac. Fluorescent nucleotide analogs were obtained from New England Nuclear (DuPont).

6. Hybridization reactions contained 1.0 μl of fluorescent cDNA synthesis product (5) and 1.0 μl of hybridization buffer [10× saline sodium citrate (SSC) and 0.2% SDS]. The 2.0-μl probe mixtures were aliquoted onto the microarray surface and covered with cover slips (12 mm round). Arrays were transferred to a hybridization chamber (3) and incubated for 18 hours at 65°C. Arrays were washed for 5 min at room temperature in low-stringency wash buffer (1× SSC and 0.1% SDS), then for 10 min at room temperature in high-stringency wash buffer (0.1× SSC and 0.1% SDS). Arrays were scanned in 0.1× SSC with the use of a fluorescence laser-scanning device (3).

7. Samples of poly(A)⁺ mRNA (4, 5) were spotted onto nylon membranes (Nytran) and crosslinked with ultraviolet light with the use of a Stratalinker 1800 (Stratagene). Probes were prepared by random priming with the use of a Prime-It II kit (Stratagene) in the presence of [³²P]dATP. Hybridizations were carried out according to the instructions of the manu-

facturer. Quantitation was performed on a Phosphorimager (Molecular Dynamics).

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Gene Therapy in Peripheral Blood Lymphocytes and Bone Marrow for ADA⁻ Immunodeficient Patients

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Adenosine deaminase (ADA) deficiency results in severe combined immunodeficiency, the first genetic disorder treated by gene therapy. Two different retroviral vectors were used to transfer ex vivo the human ADA minigene into bone marrow cells and peripheral blood lymphocytes from two patients undergoing exogenous enzyme replacement therapy. After 2 years of treatment, long-term survival of T and B lymphocytes, marrow cells, and granulocytes expressing the transferred ADA gene was demonstrated and resulted in normalization of the immune repertoire and restoration of cellular and humoral immunity. After discontinuation of treatment, T lymphocytes, derived from transduced peripheral blood lymphocytes, were progressively replaced by marrow-derived T cells in both patients. These results indicate successful gene transfer into long-lasting progenitor cells, producing a functional multilineage progeny.

Severe combined immunodeficiency associated with inherited deficiency of ADA (1) is usually fatal unless affected children are kept in protective isolation or the immune system is reconstituted by bone marrow transplantation from a human leukocyte antigen (HLA)–identical sibling donor (2). This is the therapy of choice, although it is available only for a minority of patients. In recent years, other forms of therapy have been developed, including transplants from haploidentical donors (3, 4), exogenous enzyme replacement (5), and somatic-cell gene therapy (6–9).

We previously reported a preclinical model in which ADA gene transfer and expression

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successfully restored immune functions in human ADA-deficient (ADA⁻) peripheral blood lymphocytes (PBLs) in immunodeficient mice in vivo (10, 11). On the basis of these preclinical results, the clinical application of gene therapy for the treatment of ADA⁻ SCID (severe combined immunodeficiency disease) patients who previously failed exogenous enzyme replacement therapy was approved by our Institutional Ethical Committees and by the Italian National Committee for Bioethics (12). In addition to evaluating the safety and efficacy of the gene therapy procedure, the aim of the study was to define the relative role of PBLs and hematopoietic stem cells in the long-term reconstitution of immune functions after retroviral vector–mediated ADA gene transfer. For this purpose, two structurally identical vectors expressing the human ADA complementary DNA (cDNA), distinguishable by the presence of alternative restriction sites in a nonfunctional region of the viral long-terminal repeat (LTR), were used to transduce PBLs and bone marrow (BM) cells independently. This procedure allowed identification of the origin of

GENETIC ENGINEERING NEWS

GEN

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Pharmagene Raises More Capital for Research on Human Tissues

By Sophia Fox

Pharmagene, the Royston, U.K.-based biopharmaceutical company specialising in the use of human biomaterials for drug discovery research, has raised a further £5 million from a group of investors led by 3i and Abacus Nominees. The funding will enable the company to expand both its human biomaterials collection and its capabilities across a range of proprietary platform technologies.

Gordon Baxter, Ph.D., Pharmagene's cofounder and chief operating officer, claimed "by the end of this year Pharmagene will have access to the largest collection of human RNAs and proteins anywhere in the world, and a range of innovative, yet robust technologies

SEE PHARMAGENE, P. 9

Perkin-Elmer Acquires PerSeptive to Expand Its Capabilities in Gene-Based Drug Discovery

By John Sterling

Perkin-Elmer's (PE; Norwalk, CT) decision last month to acquire PerSeptive Biosystems (Framingham, MA) via a $360 million stock swap was designed to strengthen PE in terms of broad capabilities in gene-based drug discovery. The company's main goal is to develop new products to improve the integration of genetic and protein research.

"This merger will enhance our position as an effective provider of innovative, integrated platforms enabling our customers to be more efficient and cost-effective in bringing new pharmaceuticals to market," says Tony L. White, PE's chairman, president and CEO. "The combination of our two companies should bolster our presence in the life sciences, [and it is our] belief that we must take bold action now to lead the emerging era of molecular medicine with leading positions in both genetic and protein analysis."

A driving force behind the merger is the vast amount of genet-ic information about human disease that is being accumulated by researchers and biotech companies working in the area of genomics. It is becoming increasingly obvious that these data need to be complemented with technologies for studying proteins and protein networks—a field known as proteomics (see GEN, September 1, 1997, p.1).

PE officials, who claim that MALDI-TOF (Matrix Assisted

SEE ACQUISITION, P. 10

Perkin-Elmer acquired PerSeptive Biosystems for $360 million to obtain new technologies in mass spectrometry, bioseparations and purification for product development projects, spanning the range from genomics to proteomics.

FDA OKs Genzyme's Carticel Product for Damage to Knees

By Naomi Pfeiffer
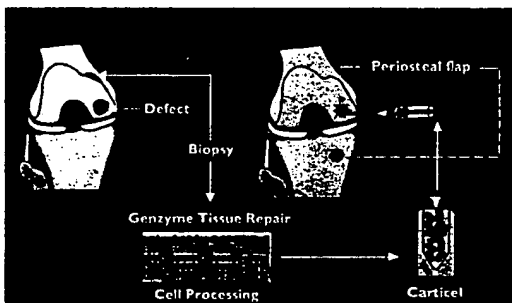
Carticel, which was approved for the repair of clinically significant, symptomatic cartilaginous defects of the femoral condyle (medial, lateral or trochlear) caused by acute or repetitive trauma, employs a proprietary process to grow autologous cartilage cells for implantation.

The FDA has approved a knee-cartilage replacement product made by Genzyme Tissue Repair (Cambridge, MA), a tracking-stock division of Genzyme Corp., for people with trauma-damaged knees.

Carticel™ (autologous cultured chondrocytes) is the first product to be licensed under the FDA's pro-

SEE GENZYME, P. 6

Strategies for Target Validation Streamline Evaluation of Leads

By Vicki Glaser

Acacia Biosciences (Richmond, CA) last month announced its first agreement with a major pharmaceutical company, signing a deal with Eli Lilly (Indianapolis, IN) to use Acacia's Genome Reporter Matrix (GRM) to select and optimize some of Lilly's lead compounds. Acacia's yeast-based system for profiling drug activity is useful for evaluating the therapeutic potential of lead compounds, and it also has a role in the identification and validation of new drug targets.

"We're using the ecosystem of a cell to allow us to deduce the mechanism of action and target for any chemical," explains Bruce Cohen, president and CEO. "We screen for every target in a cell simultaneously...using transcription as a readout for how a cell is adapting to any perturbation," he says.

The GRM technology consists of two main databases: one is the genetic response profile, showing the effects of mutations in each individual yeast gene and compensatory gene regulatory mechanisms; the other is the chemical response profile, which documents changes in gene expression in response to chemical compounds. Computational analysis and pattern matching between the genetic and chemical profiles yields information on the specificity, potency and side-effects risk of a drug lead.

Targeting Targets

No longer is mapping and sequencing a gene—or the human genome—an end unto itself, but

SEE TARGET, P. 15

Sticky Ends

Avigen received two grants from the NIH & University of California for research on gene therapy for treatment of cancer & HIV infections...MRL Pharmaceutical Services, of Reston, VA, launched the TSN Bug Finder, which is able to locate & retrieve client-specified microorganisms in real-time...Gensia Sicor, Inc. will move its corporate staff from San Diego to Irvine, CA, by end of year...

FDA accepted NDA from Sepracor for levalbuterol HCl inhalation solution...An $11.7M mezzanine financing has been closed by Activated Cell Therapy, which changed its name to Dendreon Corporation...Astra AB will build major research facility in Waltham, MA, and is also relocating Astra Arcus research facility from Rochester to Boston area...Prolifix Ltd. team used a small peptide to inhibit the E2F protein complex and induced

apoptosis in mammalian tumor cells...Vertex Pharmaceuticals, Inc. and Alpha Therapeutic Corp. ended an agreement to develop VX-366 for treatment of inherited hemoglobin disorders...NaviCyte received Phase I SBIR grant for up to $100,000 from NIH for development of prototype of its NaviFlow technology for high-throughput screening ...Covance Inc. will invest $21 million in expansion and renovation of its facility in Indianapolis, IN.
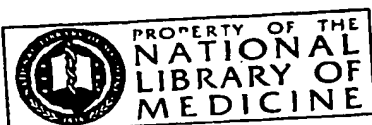
Target

merely a means to an end. The critical next step is to validate the gene and its protein product as a potential drug target. The Human Genome Project continues to produce a treasure chest of expressed sequence tags (ESTs) and a tantalizing array of complete gene sequences.

Companies are applying a variety of functional genomic strategies to link genes to specific diseases and to multigenic phenotypes. Yet the ultimate challenge for pharmaceutical companies is to sift through all the sequence and differential gene expression data to identify the best targets for drug discovery.

Spinning off technology developed at the University of North Carolina (Chapel Hill), Cytogen Corp. (Princeton, NJ) formed its wholly owned subsidiary AxCell Biosciences earlier this year. The young company is building a protein interaction database, cataloging all the interactions the modular domains of proteins can engage in with a range of ligands, in order to gain insight into protein function and to select the most critical interaction to target for drug development.

AxCell's cloning-of-ligand-targets (COLT) technology employs "recognition units" from the company's genetic diversity library (GDL) to map functional protein interactions and quantitate their affinity. The company's inter-functional protcom-ic database (IFP-dbasc) elucidates protein interaction networks and structure-activity relationships based on ligand affinity with protein modular domains.

Defining Disease Pathways

Signal Pharmaceuticals, Inc.'s (San Diego, CA) integrated drug target and discovery effort is based on mapping gene-regulating pathways in cells and identifying small molecules that regulate the activation of those genes. In collaboration with academic researchers, the company has identified a large number of regulatory proteins in several mitogen-activated protein (MAP) kinase pathways (including the JNK, FRK and p38

signaling pathways), which Signal is evaluating for the treatment of autoimmune, inflammatory, cardiovascular and neurologic diseases, and cancer. Other target identification

The Genome Reporter Matrix depicts a subset of a yeast array. Each colony harbors a GFP-reporter construct for a single gene. Collectively, the array reports the expression of all yeast genes. A: Array in visible light. B: Image of fluorescent emission from the array.
Acacia Biosciences.

programs' focus on the NF-kB pathway, estrogen-related genes and central/peripheral nervous system genes.

Regulating cytokine production in immune and inflammatory disorders,

and modifying bone metabolism to treat osteoporosis are the focus of Signal's collaboration with Tanabe Seiyaku (Osaka, Japan). Signal has partnered with Organon/Akzo Nobel (Netherlands) to identify estrogen-responsive genes as targets for treating neurodegenerative and psychiatric diseases, atherosclerosis and ischemia, and with Roche Bioscience (Palo Alto, CA) to develop human peripheral nerve cell lines for the discovery of treatments for pain and incontinence.

Exelixis' (S. San Francisco, CA) strategy for target selection is to define disease pathways and identify regulatory molecules that activate or inhibit those biochemical/genetic pathways. Based on the finding that these pathways are conserved across species, the company is studying the model genetic systems of Drosophila and *Caenorhabditis elegans*. Using its PathFinder technology, Exelixis systematically introduces mutations into the genomes of these model organisms, looking for mutations that enhance or suppress the target disease-related gene. These novel genes then become the basis of drug screening assays.

Cadus Pharmaceutical Corp. (Tarrytown, NY) is identifying surrogate ligands to newly discovered orphan G-protein coupled transmembrane receptors of unknown function to determine the suitability of the receptors as drug targets. Inserting the novel receptor in a yeast system yields a ligand that activates the receptor. Access to a surrogate ligand allows the company to screen for receptor antagonists in the yeast system.

"The antagonist plus the surrogate ligand gives you two probes—an on probe and an off probe—which allows you to look at function," explains David Webb, Ph.D., vp of research and chief scientific officer. A surrogate ligand also provides information on which G-protein interacts with the orphan receptor and its associated signaling pathways, further clarifying the role of the receptor as a potential drug target. Cadus' collaboration with SmithKline (Philadelphia) capitalizes on Cadus' ability to determine orphan receptor function, applying the technology to SmithKline's proprietary, newly discovered G-protein receptors.

Cadus' recombinant yeast system can also be used to screen cell and tissue extracts for natural ligands, and the company is accelerating its internal drug-discovery efforts in the areas of cancer, inflammation and allergy. A recent equity investment in Axiom Biotechnologies (San Diego, CA) gave Cadus a license to Axiom's high-throughput pharmacologic screening system for lead optimization and discovery.

As its name implies, gene/Networks (Alameda, CA) focuses on identifying gene networks that contribute to multigenic phenotypes and complex disease processes. The integration of mouse and human genetic studies forms the basis of the technology. The Genome Tagged Mice database in development will serve as a library of natural mouse genetic and phenotypic variation. Disease-related genes identified in mice are then evaluated in human family- and population-based studies to confirm their clinical relevance and linkages to pathophysiologic traits.

Blocking Gene Expression

Inactivating a gene known to be expressed in association with a particular disease is one approach to identifying appropriate therapeutic targets. The target validation and discovery program at **Ribozyme Pharmaceuticals, Inc.** (Boulder, CO) applies the company's ribozyme technology to achieve selective inhibition of gene expression in cell culture and in animals.

Correlation of the gene expression inhibition with phenotype can

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Target

suggest the relative importance of the gene in disease pathology. The company's nuclease-resistant ribozymes form the basis of a collaboration with Schering AG (Germany) for drug target validation and the development of ribozyme-based therapeutic agents, and with Chiron Corp. (Emeryville, CA) for target validation.

With several antisense compounds now progressing through clinical trials, the concept of using oligonucleotides to inhibit gene activity is not new. But rather than focusing on therapeutics development, Sequitur, Inc. (Natick, MA) is creating antisense compounds for the purpose of determining gene function and validating drug targets. Clients typically provide the one-year-old company with the sequence (or EST) of a potential gene target and, in return, Sequitur custom designs a series of three to six antisense compounds that yield a three-to-ten-fold inhibition of the target gene in cell culture. The company also provides oligofectins, a series of cationic lipids, to deliver the oligonucleotides to a variety of cultured cells.

"Differential expression information is just for correlation, it doesn't tell function or confirm what would be a good target," says Tod Woolf, Ph.D., director of technology development at Sequitur. Whereas, antisense compounds will inhibit a target, Sequitur offers both phosphorothioate DNA antisense compounds, and its proprietary Next Generation chimeric oligonucleotides, which have a higher hybridization affinity, greater specificity and reduced toxicity, according to the company.

Mining Pathogen Genomes

Companies such as Human Genome Sciences (HGS; Rockville, MD), Incyte (Palo Alto, CA),
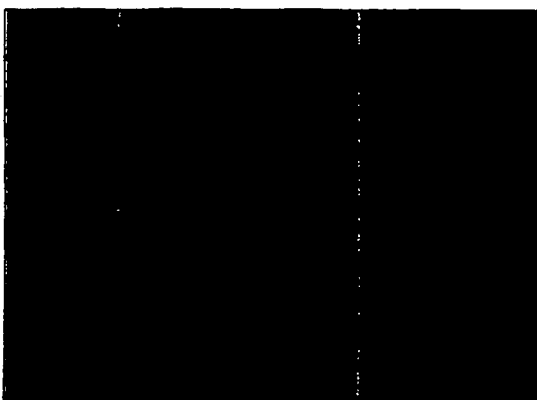
AxCell Biosciences scientists say their technology enables the rapid and simple functional identification of the two essential molecular components of protein interaction networks: specific recognition units that bind distinct modular protein domains are identified and isolated using a combination structural/functional approach that uses both peptide phase display Genetic Diversity Libraries (GDL) and bioinformatics, and cloning of Ligand Targets (COLT) technology utilizes recognition units as functional probes to isolate families of interactor proteins.

Millennium Pharmaceuticals Inc. (Cambridge, MA) and Genome Therapeutics (Waltham, MA) are relying on high-speed DNA sequencing, positional cloning and other strategies to identify specific microbial genomic sites that would be good targets for infectious disease therapeutics.

HGS recently completed sequencing of the bacterial pathogen Streptococcus pneumoniae, which is the focus of an agreement with Hoffmann-La Roche (Basel, Switzerland). Roche will use the sequence data to develop new anti-infectives against S. pneumoniae. HGS and Roche have expanded their collaboration to include a nonexclusive license to access sequence information for the intestinal bacterium Enterococcus faecalis.

Incyte Pharmaceuticals has completed one-fold coverage of the Candida albicans genome, identify-

ing 60% of the genes of this fungal pathogen. This genome will become part of the company's PathoSeq microbial database. Incyte recently introduced the ZooSeq animal gene sequence and expression database. The database will provide genomic information across various species commonly used in preclinical drug testing, which may help to better define potential drug targets.

Millennium Pharmaceuticals continues to report success in identifying novel drug targets, having recently discovered a novel chemokine called neurotactin and a new class of MAD-related proteins that inhibit transforming growth factor beta (TGF-β) signaling. The company also received U.S. patent coverage for the tub genes, believed to play a role in obesity, and for the gene that encodes the protein melastatin, which appears to suppress metastasis in malignant melanoma. ∎

Pangea

Smith, now a computer programmer, is an expert in systems integration, internet technologies and the application of industrial engineering principles to the drug discovery process. Before co-founding Pangea, he was the manager of software development at Attorney's Briefcase, a legal research software company.

By being "in the trenches" with customers and collaborators, Bellenson and Smith sensed the frustration of pharmaceutical researchers whose incompatible tools have impeded their progress. According to Bellenson, "Most of them are geared toward analyzing one molecule at a time. It's like emptying the ocean with an eye dropper—an incompatible eye dropper at that. A pharmaceutical company may have 30 different drug discovery teams with various approaches. The problem is to manage the process of experimenting with a lot of different approaches, to automate while maintaining flexibility."

GeneWorld 2.1 enables "integration of the entire target discovery and validation process," Bellenson says. The commercial software package coordinates the entire process of sequence-data analysis and can be integrated with other programs and databases, according to Smith, who adds that it handles thousands of sequence results, organizes and automates annotation and seamlessly interacts with growing genome databases. Simple forms and menus enable users to turn raw sequence data into crucial knowledge for drug discovery by applying algorithms to sequences, creating custom analysis strategies and producing useful reports, without the need for writing computer code. GeneWorld 2.1 runs on a variety of platforms and operating systems.

Pairing industrial relational database-management systems with a web-browser interface, Pangea's Operating System of Drug Discovery" is an open-computing framework that allows client/server and Java-enabled web-based technologies to collect, organize and analyze drug discovery information for pharmaceutical companies to simplify and accelerate drug discovery. The technology unites automated genomics database analysis for drug target site selection, chemical information database analysis and large-scale combinatorial chemistry project management and high-throughput screening project management for drug lead efficacy analysis. Pangea officials maintain that these integrated elements provide a unified environment for chemists, biologists and others involved in the drug discovery process to work together with

Europe

GTAC Chairman, Professor Norman C. Nevin, said 1996 saw "four important developments": an increase in enquiries and submissions made to GTAC; an increase in the complexity of submitted protocols; a continuing shift from gene therapy for single-gene disorders toward strategies aimed at tumour destruction in cancer; and a growth in international sponsorship of U.K. gene therapy trials.

Since 1993, GTAC and its predecessor, the Clothier Committee, have approved 18 U.K. gene therapy clinical trials (13 of which have been carried out), which are listed in the report. The disease areas targeted by these trials include severe combined immunodeficiency (1 trial), cystic fibrosis (6), metastatic melanoma (2), lymphoma (2), neuroblastoma (1), breast cancer (1), Hurler's syndrome (1), cervical cancer (1), glioblastoma

Bioinformaticists can design and save Strategies, such as the one shown here, that forward data through multiple-step analyses logically and automatically. Researchers throughout your organization can apply the same Strategies to their own data.

commercial and public domain software.

Pangea's Operating System of Drug Discovery can accommodate Sybase, Oracle or Informix relational database-management systems and any version of UNIX. It absorbs new data formats, databases, algorithms and analysis paradigms into the automated workflow without software modifications. Netscape Navigator" provides a friendly user interface from PC, Macintosh, and UNIX workstations.

In the near term, Pangea plans to complete its bioinformatics core with two more programs. Gene Foundry, a sample tracking and workflow sequence package for DNA sequence and fragment information, will also offer interaction with robots, reagent tracking and troubleshooting. Gene Thesaurus, the other package is a "warehouse of bioinformatics data," says Bellenson. ∎

breast cancer, breast cancer with liver metastases, glioblastoma, malignant ascites due to gastrointestinal cancer and ovarian cancer.

Copies of the GTAC third annual report are available from the GTAC Secretariat, Wellington House, 133-155 Waterloo Road, London SE1 8UG, U.K.

Coated Lenses Prevent PCO

Scientists in the U.K. say it may be possible to prevent posterior capsule opacification (PCO), a common complication following cataract surgery, by using the implanted polymethylmethacrylate (PMMA) intraocular lens as a drug delivery system. PCO occurs in 30-50% of cataract surgery patients as a result of stimulated cell growth within the remaining capsular bag. The condition causes a decline in visual acuity and requires expensive laser treatment, thus negating the routine use of cataract surgery in underdeveloped countries, explains G. Duncan, at the

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Exploring the Metabolic and Genetic Control of Gene Expression on a Genomic Scale

Joseph L. DeRisi, Vishwanath R. Iyer, Patrick O. Brown*

DNA microarrays containing virtually every gene of *Saccharomyces cerevisiae* were used to carry out a comprehensive investigation of the temporal program of gene expression accompanying the metabolic shift from fermentation to respiration. The expression profiles observed for genes with known metabolic functions pointed to features of the metabolic reprogramming that occur during the diauxic shift, and the expression patterns of many previously uncharacterized genes provided clues to their possible functions. The same DNA microarrays were also used to identify genes whose expression was affected by deletion of the transcriptional co-repressor *TUP1* or overexpression of the transcriptional activator *YAP1*. These results demonstrate the feasibility and utility of this approach to genomewide exploration of gene expression patterns.

The complete sequences of nearly a dozen microbial genomes are known, and in the next several years we expect to know the complete genome sequences of several metazoans, including the human genome. Defining the role of each gene in these genomes will be a formidable task, and understanding how the genome functions as a whole in the complex natural history of a living organism presents an even greater challenge.

Knowing when and where a gene is expressed often provides a strong clue as to its biological role. Conversely, the pattern of genes expressed in a cell can provide detailed information about its state. Although regulation of protein abundance in a cell is by no means accomplished solely by regulation of mRNA, virtually all differences in cell type or state are correlated with changes in the mRNA levels of many genes. This is fortuitous because the only specific reagent required to measure the abundance of the mRNA for a specific gene is a cDNA sequence. DNA microarrays, consisting of thousands of individual gene sequences printed in a high-density array on a glass microscope slide (*1*, *2*), provide a practical and economical tool for studying gene expression on a very large scale (*3*–*6*).

Saccharomyces cerevisiae is an especially

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favorable organism in which to conduct a systematic investigation of gene expression. The genes are easy to recognize in the genome sequence, *cis* regulatory elements are generally compact and close to the transcription units, much is already known about its genetic regulatory mechanisms, and a powerful set of tools is available for its analysis.

A recurring cycle in the natural history of yeast involves a shift from anaerobic (fermentation) to aerobic (respiration) metabolism. Inoculation of yeast into a medium rich in sugar is followed by rapid growth fueled by fermentation, with the production of ethanol. When the fermentable sugar is exhausted, the yeast cells turn to ethanol as a carbon source for aerobic growth. This switch from anaerobic growth to aerobic respiration upon depletion of glucose, referred to as the diauxic shift, is correlated with widespread changes in the expression of genes involved in fundamental cellular processes such as carbon metabolism, protein synthesis, and carbohydrate storage (*7*). We used DNA microarrays to characterize the changes in gene expression that take place during this process for nearly the entire genome, and to investigate the genetic circuitry that regulates and executes this program.

Yeast open reading frames (ORFs) were amplified by the polymerase chain reaction (PCR), with a commercially available set of primer pairs (*8*). DNA microarrays, containing approximately 6400 distinct DNA sequences, were printed onto glass slides by

using a simple robotic printing device (*9*). Cells from an exponentially growing culture of yeast were inoculated into fresh medium and grown at 30°C for 21 hours. After an initial 9 hours of growth, samples were harvested at seven successive 2-hour intervals, and mRNA was isolated (*10*). Fluorescently labeled cDNA was prepared by reverse transcription in the presence of Cy3(green)- or Cy5(red)-labeled deoxyuridine triphosphate (dUTP) (*11*) and then hybridized to the microarrays (*12*). To maximize the reliability with which changes in expression levels could be discerned, we labeled cDNA prepared from cells at each successive time point with Cy5, then mixed it with a Cy3-labeled "reference" cDNA sample prepared from cells harvested at the first interval after inoculation. In this experimental design, the relative fluorescence intensity measured for the Cy3 and Cy5 fluors at each array element provides a reliable measure of the relative abundance of the corresponding mRNA in the two cell populations (Fig. 1). Data from the series of seven samples (Fig. 2), consisting of more than 43,000 expression-ratio measurements, were organized into a database to facilitate efficient exploration and analysis of the results. This database is publicly available on the Internet (*13*).

During exponential growth in glucose-rich medium, the global pattern of gene expression was remarkably stable. Indeed, when gene expression patterns between the first two cell samples (harvested at a 2-hour interval) were compared, mRNA levels differed by a factor of 2 or more for only 19 genes (0.3%), and the largest of these differences was only 2.7-fold (*14*). However, as glucose was progressively depleted from the growth media during the course of the experiment, a marked change was seen in the global pattern of gene expression. mRNA levels for approximately 710 genes were induced by a factor of at least 2, and the mRNA levels for approximately 1030 genes declined by a factor of at least 2. Messenger RNA levels for 183 genes increased by a factor of at least 4, and mRNA levels for 203 genes diminished by a factor of at least 4. About half of these differentially expressed genes have no currently recognized function and are not yet named. Indeed, more than 400 of the differentially expressed genes have no apparent homology

to any gene whose function is known (15). The responses of these previously uncharacterized genes to the diauxic shift therefore provides the first small clue to their possible roles.

The global view of changes in expression of genes with known functions provides a vivid picture of the way in which the cell adapts to a changing environment. Figure 3 shows a portion of the yeast metabolic pathways involved in carbon and energy metabolism. Mapping the changes we observed in the mRNAs encoding each enzyme onto this framework allowed us to infer the redirection in the flow of metabolites through this system. We observed large inductions of the genes coding for the enzymes aldehyde dehydrogenase (ALD2) and acetyl-coenzyme A (CoA) synthase (ACS1), which function together to convert the products of alcohol dehydrogenase into acetyl-CoA, which in turn is used to fuel the tricarboxylic acid (TCA) cycle and the glyoxylate cycle. The concomitant shutdown of transcription of the genes encoding pyruvate decarboxylase and induction of pyruvate carboxylase rechannels pyruvate away from acetaldehyde, and instead to oxalacetate, where it can serve to supply the TCA cycle and gluconeogenesis. Induction of the pivotal genes PCK1, encoding phosphoenolpyruvate carboxykinase, and FBP1, encoding fructose 1,6-biphosphatase, switches the directions of two key irreversible steps in glycolysis, reversing the flow of metabolites along the reversible steps of the glycolytic pathway toward the essential biosynthetic precursor, glucose-6-phosphate. Induction of the genes coding for the trehalose synthase and glycogen synthase complexes promotes channeling of glucose-6-phosphate into these carbohydrate storage pathways.

Just as the changes in expression of genes encoding pivotal enzymes can provide insight into metabolic reprogramming, the behavior of large groups of functionally related genes can provide a broad view of the systematic way in which the yeast cell adapts to a changing environment (Fig. 4). Several classes of genes, such as cytochrome c–related genes and those involved in the TCA/glyoxylate cycle and carbohydrate storage, were coordinately induced by glucose exhaustion. In contrast, genes devoted to protein synthesis, including ribosomal proteins, tRNA synthetases, and translation, elongation, and initiation factors, exhibited a coordinated decrease in expression. More than 95% of ribosomal genes showed at least twofold decreases in expression during the diauxic shift (Fig. 4) (13). A noteworthy and illuminating exception was that the

genes encoding mitochondrial ribosomal genes were generally induced rather than repressed after glucose limitation, highlighting the requirement for mitchondrial biogenesis (13). As more is learned about the functions of every gene in the yeast genome, the ability to gain insight into a cell's response to a changing environment through its global gene expression patterns will become increasingly powerful.

Several distinct temporal patterns of expression could be recognized, and sets of genes could be grouped on the basis of the similarities in their expression patterns. The characterized members of each of these groups also shared important similarities in their functions. Moreover, in most cases, common regulatory mechanisms could be inferred for sets of genes with similar expression profiles. For example, seven genes showed a late induction profile, with mRNA levels increasing by more than ninefold at

the last timepoint but less than threefold at the preceding timepoint (Fig. 5B). All of these genes were known to be glucose-repressed, and five of the seven were previously noted to share a common upstream activating sequence (UAS), the carbon source response element (CSRE) (16–20). A search in the promoter regions of the remaining two genes, ACR1 and IDP2, revealed that ACR1, a gene essential for ACS1 activity, also possessed a consensus CSRE motif, but interestingly, IDP2 did not. A search of the entire yeast genome sequence for the consensus CSRE motif revealed only four additional candidate genes, none of which showed a similar induction.

Examples from additional groups of genes that shared expression profiles are illustrated in Fig. 5, C through F. The sequences upstream of the named genes in Fig. 5C all contain stress response elements (STRE), and with the exception

Fig. 1. Yeast genome microarray. The actual size of the microarray is 18 mm by 18 mm. The microarray was printed as described (9). This image was obtained with the same fluorescent scanning confocal microscope used to collect all the data we report (49). A fluorescently labeled cDNA probe was prepared from mRNA isolated from cells harvested shortly after inoculation (culture density of $<5 \times 10^6$ cells/ml and media glucose level of 19 g/liter) by reverse transcription in the presence of Cy3-dUTP. Similarly, a second probe was prepared from mRNA isolated from cells taken from the same culture 9.5 hours later (culture density of $\sim 2 \times 10^8$ cells/ml, with a glucose level of <0.2 g/liter) by reverse transcription in the presence of Cy5-dUTP. In this image, hybridization of the Cy3-dUTP–labeled cDNA (that is, mRNA expression at the initial timepoint) is represented as a green signal, and hybridization of Cy5-dUTP–labeled cDNA (that is, mRNA expression at 9.5 hours) is represented as a red signal. Thus, genes induced or repressed after the diauxic shift appear in this image as red and green spots, respectively. Genes expressed at roughly equal levels before and after the diauxic shift appear in this image as yellow spots.

of HSP42, have previously been shown to be controlled at least in part by these elements (21–24). Inspection of the sequences upstream of HSP42 and the two uncharacterized genes shown in Fig. 5C, YKL026c, a hypothetical protein with similarity to glutathione peroxidase, and YGR043c, a putative transaldolase, revealed that each of these genes also possess repeated upstream copies of the stress-responsive CCCCT motif. Of the 13 additional genes in the yeast genome that shared this expression profile [including HSP30, ALD2, OM45, and 10 uncharacterized ORFs (25)], nine contained one or more recognizable STRE sites in their upstream regions.

The heterotrimeric transcriptional activator complex HAP2,3,4 has been shown to be responsible for induction of several genes important for respiration (26–28). This complex binds a degenerate consensus sequence known as the CCAAT box (26). Computer analysis, using the consensus sequence TNRYTGGB (29), has suggested that a large number of genes involved in respiration may be specific targets of HAP2,3,4 (30). Indeed, a putative HAP2,3,4 binding site could be found in the sequences upstream of each of the seven cytochrome c–related genes that showed the greatest magnitude of induction (Fig. 5D). Of 12 additional cytochrome c–related genes that were induced, HAP2,3,4 binding sites were present in all but one. Significantly, we found that transcription of HAP4 itself was induced nearly ninefold concomitant with the diauxic shift.

Control of ribosomal protein biogenesis is mainly exerted at the transcriptional level, through the presence of a common upstream-activating element (UAS_rpg) that is recognized by the Rap1 DNA-binding protein (31, 32). The expression profiles of seven ribosomal proteins are shown in Fig. 5F. A search of the sequences upstream of all seven genes revealed consensus Rap1-binding motifs (33). It has been suggested that declining Rap1 levels in the cell during starvation may be responsible for the decline in ribosomal protein gene expression (34). Indeed, we observed that the abundance of RAP1 mRNA diminished by 4.4-fold, at about the time of glucose exhaustion.

Of the 149 genes that encode known or putative transcription factors, only two, HAP4 and SIP4, were induced by a factor of more than threefold at the diauxic shift. SIP4 encodes a DNA-binding transcriptional activator that has been shown to interact with Snf1, the "master regulator" of glucose repression (35). The eightfold induction of SIP4 upon depletion of glucose strongly suggests a role in the induction of

downstream genes at the diauxic shift.

Although most of the transcriptional responses that we observed were not previously known, the responses of many genes during the diauxic shift have been described. Comparison of the results we obtained by DNA microarray hybridization with previously reported results therefore provided a strong test of the sensitivity and accuracy of this approach. The expression patterns we observed for previously characterized genes showed almost perfect concordance with previously published results (36). Moreover, the differential expression measurements obtained by DNA microarray hybridization were reproducible in duplicate experiments. For example, the remarkable changes in gene expression between cells harvested immediately after inoculation and immediately after the diauxic shift (the first and sixth intervals in this time series) were measured in duplicate, independent DNA microarray hybridizations. The correlation coefficient for two complete sets of expression ratio measurements was 0.87, and for more than 95% of the genes, the expres-

Fig. 2. The section of the array indicated by the gray box in Fig. 1 is shown for each of the experiments described here. Representative genes are labeled. In each of the arrays used to analyze gene expression during the diauxic shift, red spots represent genes that were induced relative to the initial timepoint, and green spots represent genes that were repressed relative to the initial timepoint. In the arrays used to analyze the effects of the tup1Δ mutation and YAP1 overexpression, red spots represent genes whose expression was increased, and green spots represent genes whose expression was decreased by the genetic modification. Note that distinct sets of genes are induced and repressed in the different experiments. The complete images of each of these arrays can be viewed on the Internet (13). Cell density as measured by optical density (OD) at 600 nm was used to measure the growth of the culture.

sion ratios measured in these duplicate experiments differed by less than a factor of 2. However, in a few cases, there were discrepancies between our results and previous results, pointing to technical limitations that will need to be addressed as DNA microarray technology advances (37, 38). Despite the noted exceptions, the high concordance between the results we obtained in these experiments and those of previous studies provides confidence in the reliability and thoroughness of the survey.

The changes in gene expression during this diauxic shift are complex and involve integration of many kinds of information about the nutritional and metabolic state of the cell. The large number of genes whose expression is altered and the diversity of temporal expression profiles observed in this experiment highlight the challenge of understanding the underlying regulatory mechanisms. One approach to defining the contributions of individual regulatory genes to a complex program of this kind is to use DNA microarrays to identify genes whose expression is affected

Growth OD 0.14 | Growth OD 0.46 | Growth OD 0.8
Growth OD 1.8 | Growth OD 3.7 | Growth OD 6.9
Growth OD 7.3 | Δ tup1 | YAP1 +++

by mutations in each putative regulatory gene. As a test of this strategy, we analyzed the genomewide changes in gene expression that result from deletion of the *TUP1* gene. Transcriptional repression of many genes by glucose requires the DNA-binding repressor

Mig1 and is mediated by recruiting the transcriptional co-repressors Tup1 and Cyc8/Ssn6 (*39*). Tup1 has also been implicated in repression of oxygen-regulated, mating-type–specific, and DNA-damage–inducible genes (*40*).

Wild-type yeast cells and cells bearing a deletion of the *TUP1* gene (*tup1*Δ) were grown in parallel cultures in rich medium containing glucose as the carbon source. Messenger RNA was isolated from exponentially growing cells from the two populations and used to prepare cDNA labeled with Cy3 (green) and Cy5 (red), respectively (*11*). The labeled probes were mixed and simultaneously hybridized to the microarray. Red spots on the microarray therefore represented genes whose transcription was induced in the *tup1*Δ strain, and thus presumably repressed by Tup1 (*41*). A representative section of the microarray (Fig. 2, bottom middle panel) illustrates that the genes whose expression was affected by the *tup1*Δ mutation, were, in general, distinct from those induced upon glucose exhaustion [complete images of all the arrays shown in Fig. 2 are available on the Internet (*13*)]. Nevertheless, 34 (10%) of the genes that were induced by a factor of at least 2 after the diauxic shift were similarly induced by deletion of *TUP1*, suggesting that these genes may be subject to *TUP1*-mediated repression by glucose. For example, *SUC2*, the gene encoding invertase, and all five hexose transporter genes that were induced during the course of the diauxic shift were similarly induced, in duplicate experiments, by the deletion of *TUP1*.

The set of genes affected by Tup1 in this experiment also included α-glucosidases, the mating-type–specific genes *MFA1* and *MFA2*, and the DNA damage–inducible *RNR2* and *RNR4*, as well as genes involved in flocculation and many genes of unknown function. The hybridization signal corresponding to expression of *TUP1* itself was also severely reduced because of the (incomplete) deletion of the transcription unit in the *tup1*Δ strain, providing a positive control in the experiment (*42*).

Many of the transcriptional targets of Tup1 fell into sets of genes with related biochemical functions. For instance, although only about 3% of all yeast genes appeared to be *TUP1*-repressed by a factor of more than 2 in duplicate experiments under these conditions, 6 of the 13 genes that have been implicated in flocculation (*15*) showed a reproducible increase in expression of at least twofold when *TUP1* was deleted. Another group of related genes that appeared to be subject to *TUP1* repression encodes the serine-rich cell wall mannoproteins, such as Tip1 and Tir1/Srp1 which are induced by cold shock and other stresses (*43*), and similar, serine-poor proteins, the seripauperins (*44*). Messenger RNA levels for 23 of the 26 genes in this group were reproducibly elevated by at least 2.5-fold in the *tup1*Δ

Fig. 3. Metabolic reprogramming inferred from global analysis of changes in gene expression. Only key metabolic intermediates are identified. The yeast genes encoding the enzymes that catalyze each step in this metabolic circuit are identified by name in the boxes. The genes encoding succinyl-CoA synthase and glycogen-debranching enzyme have not been explicitly identified, but the ORFs YGR244 and YPR184 show significant homology to known succinyl-CoA synthase and glycogen-debranching enzymes, respectively, and are therefore included in the corresponding steps in this figure. Red boxes with white lettering identify genes whose expression increases in the diauxic shift. Green boxes with dark green lettering identify genes whose expression diminishes in the diauxic shift. The magnitude of induction or repression is indicated for these genes. For multimeric enzyme complexes, such as succinate dehydrogenase, the indicated fold-induction represents an unweighted average of all the genes listed in the box. Black and white boxes indicate no significant differential expression (less than twofold). The direction of the arrows connecting reversible enzymatic steps indicate the direction of the flow of metabolic intermediates, inferred from the gene expression pattern, after the diauxic shift. Arrows representing steps catalyzed by genes whose expression was strongly induced are highlighted in red. The broad gray arrows represent major increases in the flow of metabolites after the diauxic shift, inferred from the indicated changes in gene expression.

strain, and 18 of these genes were induced by more than sevenfold when *TUP1* was deleted. In contrast, none of 83 genes that could be classified as putative regulators of the cell division cycle were induced more than twofold by deletion of *TUP1*. Thus, despite the diversity of the regulatory systems that employ Tup1, most of the genes that it regulates under these conditions fall into a limited number of distinct functional classes.

Because the microarray allows us to monitor expression of nearly every gene in yeast, we can, in principle, use this approach to identify all the transcriptional targets of a regulatory protein like Tup1. It is important to note, however, that in any single experiment of this kind we can only recognize those target genes that are normally repressed (or induced) under the conditions of the experiment. For instance, the experiment described here analyzed a *MAT* α strain in which *MFA1* and *MFA2*, the genes encoding the a-factor mating pheromone precursor, are normally repressed. In the isogenic *tup1*Δ strain, these genes were inappropriately expressed, reflecting the role that Tup1 plays in their repression. Had we instead carried out this experiment with a *MATA* strain (in which expression of *MFA1* and *MFA2* is not repressed), it would not have been possible to conclude anything regarding the role of Tup1 in the repression of these genes. Conversely, we cannot distinguish indirect effects of the chronic absence of Tup1 in the mutant strain from effects directly attributable to its participation in repressing the transcription of a gene.

Another simple route to modulating the activity of a regulatory factor is to overexpress the gene that encodes it. *YAP1* encodes a DNA-binding transcription factor belonging to the b-zip class of DNA-binding proteins. Overexpression of *YAP1* in yeast confers increased resistance to hydrogen peroxide, o-phenanthroline, heavy metals, and osmotic stress (*45*). We analyzed differential gene expression between a wild-type strain bearing a control plasmid and a strain with a plasmid expressing *YAP1* under the control of the strong *GAL1-10* promoter, both grown in galactose (that is, a condition that induces *YAP1* overexpression). Complementary DNA from the control and *YAP1* overexpressing strains, labeled with Cy3 and Cy5, respectively, was prepared from mRNA isolated from the two strains and hybridized to the microarray. Thus, red spots on the array represent genes that were induced in the strain overexpressing *YAP1*.

Of the 17 genes whose mRNA levels increased by more than threefold when

YAP1 was overexpressed in this way, five bear homology to aryl-alcohol oxidoreductases (Fig. 2 and Table 1). An additional four of the genes in this set also belong to the general class of dehydrogenases/oxidoreductases. Very little is known about the role of aryl-alcohol oxidoreductases in *S. cerevisiae*, but these enzymes have been isolated from ligninolytic fungi, in which they participate in coupled redox reactions, oxidizing aromatic, and aliphatic unsaturated alcohols to aldehydes with the production of hydrogen peroxide (*46, 47*). The fact that a remarkable fraction of the targets identified in this experiment belong to the same small, functional group of oxidoreductases suggests that these genes

might play an important protective role during oxidative stress. Transcription of a small number of genes was reduced in the strain overexpressing Yap1. Interestingly, many of these genes encode sugar permeases or enzymes involved in inositol metabolism.

We searched for Yap1-binding sites (TTACTAA or TGACTAA) in the sequences upstream of the target genes we identified (*48*). About two-thirds of the genes that were induced by more than threefold upon Yap1 overexpression had one or more binding sites within 600 bases upstream of the start codon (Table 1), suggesting that they are directly regulated by Yap1. The absence of canonical Yap1-bind-

Fig. 4. Coordinated regulation of functionally related genes. The curves represent the average induction or repression ratios for all the genes in each indicated group. The total number of genes in each group was as follows: ribosomal proteins, 112; translation elongation and initiation factors, 25; tRNA synthetases (excluding mitochondial synthetases), 17; glycogen and trehalose synthesis and degradation, 15; cytochrome c oxidase and reductase proteins, 19; and TCA- and glyoxylate-cycle enzymes, 24.

Table 1. Genes induced by *YAP1* overexpression. This list includes all the genes for which mRNA levels increased by more than twofold upon *YAP1* overexpression in both of two duplicate experiments, and for which the average increase in mRNA level in the two experiments was greater than threefold (*50*). Positions of the canonical Yap1 binding sites upstream of the start codon, when present, and the average fold-increase in mRNA levels measured in the two experiments are indicated.

ORF	Distance of Yap1 site from ATG	Gene	Description	Fold-increase
YNL331C			Putative aryl-alcohol reductase	12.9
YKL071W	162–222 (5 sites)		Similarity to bacterial csgA protein	10.4
YML007W		YAP1	Transcriptional activator involved in oxidative stress response	9.8
YFL056C	223, 242		Homology to aryl-alcohol dehydrogenases	9.0
YLL060C	98		Putative glutathione transferase	7.4
YOL165C	266		Putative aryl-alcohol dehydrogenase (NADP+)	7.0
YCR107W			Putative aryl-alcohol reductase	6.5
YML116W	409	ATR1	Aminotriazole and 4-nitroquinoline resistance protein	6.5
YBR008C	142, 167, 364		Homology to benomyl/methotrexate resistance protein	6.1
YCLX08C			Hypothetical protein	6.1
YJR155W			Putative aryl-alcohol dehydrogenase	6.0
YPL171C	148, 212	OYE3	NAPDH dehydrogenase (old yellow enzyme), isoform 3	5.8
YLR460C	167, 317		Homology to hypothetical proteins YCR102c and YNL134c	4.7
YKR076W	178		Homology to hypothetical protein YMR251w	4.5
YHR179W	327	OYE2	NAD(P)H oxidoreductase (old yellow enzyme), isoform 1	4.1
YML131W	507		Similarity to A. thaliana zeta-crystallin homolog	3.7
YOL126C		MDH2	Malate dehydrogenase	3.3

ing sites upstream of the others may reflect an ability of Yap1 to bind sites that differ from the canonical binding sites, perhaps in cooperation with other factors, or less likely, may represent an indirect effect of Yap1 overexpression, mediated by one or more intermediary factors. Yap1 sites were found only four times in the corresponding region of an arbitrary set of 30 genes that were not differentially regulated by Yap1.

Use of a DNA microarray to characterize the transcriptional consequences of mutations affecting the activity of regulatory molecules provides a simple and powerful approach to dissection and characterization of regulatory pathways and net-

works. This strategy also has an important practical application in drug screening. Mutations in specific genes encoding candidate drug targets can serve as surrogates for the ideal chemical inhibitor or modulator of their activity. DNA microarrays can be used to define the resulting signature pattern of alterations in gene expression, and then subsequently used in an assay to screen for compounds that reproduce the desired signature pattern.

DNA microarrays provide a simple and economical way to explore gene expression patterns on a genomic scale. The hurdles to extending this approach to any other organism are minor. The equipment

required for fabricating and using DNA microarrays (9) consists of components that were chosen for their modest cost and simplicity. It was feasible for a small group to accomplish the amplification of more than 6000 genes in about 4 months and, once the amplified gene sequences were in hand, only 2 days were required to print a set of 110 microarrays of 6400 elements each. Probe preparation, hybridization, and fluorescent imaging are also simple procedures. Even conceptually simple experiments, as we described here, can yield vast amounts of information. The value of the information from each experiment of this kind will progressively increase as more is learned about the functions of each gene and as additional experiments define the global changes in gene expression in diverse other natural processes and genetic perturbations. Perhaps the greatest challenge now is to develop efficient methods for organizing, distributing, interpreting, and extracting insights from the large volumes of data these experiments will provide.

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8. Primers for each known or predicted protein coding sequence were supplied by Research Genetics. PCR was performed with the protocol supplied by Research Genetics, using genomic DNA from yeast strain S288C as a template. Each PCR product was verified by agarose gel electrophoresis and was deemed correct if the lane contained a single band of appropriate mobility. Failures were marked as such in the database. The overall success rate for a single-pass amplification of 6116 ORFs was ~94.5%.
9. Glass slides (Gold Seal) were cleaned for 2 hours in a solution of 2 N NaOH and 70% ethanol. After rinsing in distilled water, the slides were then treated with a 1:5 dilution of poly-L-lysine adhesive solution (Sigma) for 1 hour, and then dried for 5 min at 40°C in a vacuum oven. DNA samples from 100-µl PCR reactions were purified by ethanol purification in 96-well microtiter plates. The resulting precipitates were resuspended in 3× standard saline citrate (SSC) and transferred to new plates for arraying. A custom-built arraying robot was used to print on a batch of 110 slides. Details of the design of the microarrayer are available at cmgm.stanford.edu/pbrown. After printing, the microarrays were rehydrated for 30 s in a humid chamber and then snap-dried for 2 s on a hot plate (100°C). The DNA was then ultraviolet (UV)-crosslinked to the surface by subjecting the slides to 60 mJ of energy (Stratagene Stratalinker). The rest of the poly-L-lysine surface was blocked by a 15-min incubation in a solution of 70 mM succinic anhydride dissolved in a solution consisting of 315 ml of 1-methyl-2-pyrrolidinone (Aldrich) and 35 ml of 1 M boric acid (pH 8.0). Directly after the blocking reac-

Fig. 5. Distinct temporal patterns of induction or repression help to group genes that share regulatory properties. **(A)** Temporal profile of the cell density, as measured by OD at 600 nm and glucose concentration in the media. **(B)** Seven genes exhibited a strong induction (greater than ninefold) only at the last timepoint (20.5 hours). With the exception of *IDP2*, each of these genes has a CSRE UAS. There were no additional genes observed to match this profile. **(C)** Seven members of a class of genes marked by early induction with a peak in mRNA levels at 18.5 hours. Each of these genes contain STRE motif repeats in their upstream promoter regions. **(D)** Cytochrome c oxidase and ubiquinol cytochrome c reductase genes. Marked by an induction coincident with the diauxic shift, each of these genes contains a consensus binding motif for the HAP2,3,4 protein complex. At least 17 genes shared a similar expression profile. **(E)** *SAM1*, *GPP1*, and several genes of unknown function are repressed before the diauxic shift, and continue to be repressed upon entry into stationary phase. **(F)** Ribosomal protein genes comprise a large class of genes that are repressed upon depletion of glucose. Each of the genes profiled here contains one or more RAP1-binding motifs upstream of its promoter. RAP1 is a transcriptional regulator of most ribosomal proteins.

tion, the bound DNA was denatured by a 2-min incubation in distilled water at ~95°C. The slides were then transferred into a bath of 100% ethanol at room temperature, rinsed, and then spun dry in a clinical centrifuge. Slides were stored in a closed box at room temperature until used.

10. YPD medium (8 liters), in a 10-liter fermentation vessel, was inoculated with 2 ml of a fresh overnight culture of yeast strain DBY7286 (MATa, ura3, GAL2). The fermentor was maintained at 30°C with constant agitation and aeration. The glucose content of the media was measured with a UV test kit (Boehringer Mannheim, catalog number 716251). Cell density was measured by OD at 600-nm wavelength. Aliquots of culture were rapidly withdrawn from the fermentation vessel by peristaltic pump, spun down at room temperature, and then flash frozen with liquid nitrogen. Frozen cells were stored at −80°C.

11. Cy3-dUTP or Cy5-dUTP (Amersham) was incorporated during reverse transcription of 1.25 μg of polyadenylated [poly(A)+] RNA, primed by a dT(16) oligomer. This mixture was heated to 70°C for 10 min, and then transferred to ice. A premixed solution, consisting of 200 U Superscript II (Gibco), buffer, deoxyribonucleoside triphosphates, and fluorescent nucleotides, was added to the RNA. Nucleotides were used at these final concentrations: 500 μM for dATP, dCTP, and dGTP and 200 μM for dTTP. Cy3-dUTP and Cy5-dUTP were used at a final concentration of 100 μM. The reaction was then incubated at 42°C for 2 hours. Unincorporated fluorescent nucleotides were removed by first diluting the reaction mixture with of 470 μl of 10 mM tris-HCl (pH 8.0)/1 mM EDTA and then subsequently concentrating the mix to ~5 μl, using Centricon-30 microconcentrators (Amicon).

12. Purified, labeled cDNA was resuspended in 11 μl of 3.5× SSC containing 10 μg poly(dA) and 0.3 μl of 10% SDS. Before hybridization, the solution was boiled for 2 min and then allowed to cool to room temperature. The solution was applied to the microarray under a cover slip, and the slide was placed in a custom hybridization chamber which was subsequently incubated for ~8 to 12 hours in a water bath at 62°C. Before scanning, slides were washed in 2× SSC, 0.2% SDS for 5 min, and then 0.05× SSC for 1 min. Slides were dried before scanning by centrifugation at 500 rpm in a Beckman CS-6R centrifuge.

13. The complete data set is available on the Internet at cmgm.stanford.edu/pbrown/explore/index.html

14. For 95% of all the genes analyzed, the mRNA levels measured in cells harvested at the first and second interval after inoculation differed by a factor of less than 1.5. The correlation coefficient for the comparison between mRNA levels measured for each gene in these two different mRNA samples was 0.98. When duplicate mRNA preparations from the same cell sample were compared in the same way, the correlation coefficient between the expression levels measured for the two samples by comparative hybridization was 0.99.

15. The numbers and identities of known and putative genes, and their homologies to other genes, were gathered from the following public databases: Saccharomyces Genome Database (genome-www. stanford.edu), Yeast Protein Database (quest7. proteome.com), and Munich Information Centre for Protein Sequences (speedy.mips.biochem.mpg.de/ mips/yeast/index.htmlx).

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38. Cross-hybridization of highly related sequences can also occasionally obscure changes in gene expression, an important concern where members of gene families are functionally specialized and differentially regulated. The major alcohol dehydrogenase genes, ADH1 and ADH2, share 88% nucleotide identity. Reciprocal regulation of these genes is an important feature of the diauxic shift, but was not observed in this experiment, presumably because of cross-hybridization of the fluorescent cDNAs representing these two genes. Nevertheless, we were able to detect differential expression of closely related isoforms of other enzymes, such as HXK1/HXK2 (77% identical) [P. Herrero et al., Yeast 11, 137 (1995)], MLS1/ DAL7 (73% identical) (20), and PGM1/PGM2 (72% identical) [D. Oh, J. E. Hopper, Mol. Cell. Biol. 10, 1415 (1990)], in accord with previous studies. Use in the microarray of deliberately selected DNA sequences corresponding to the most divergent segments of homologous genes, in lieu of the complete gene sequences, should relieve this problem in many cases.

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dance between the sets of genes that appeared to be induced was very high between the two experiments. When only the 355 genes that showed at least a twofold increase in mRNA in the tup1Δ strain in either of the duplicate experiments were compared, the correlation coefficient was 0.82.

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49. Microarrays were scanned using a custom-built scanning laser microscope built by S. Smith with software written by N. Ziv. Details concerning scanner design and construction are available at cmgm. stanford.edu/pbrown. Images were scanned at a resolution of 20 μm per pixel. A separate scan, using the appropriate excitation line, was done for each of the two fluorophores used. During the scanning process, the ratio between the signals in the two channels was calculated for several array elements containing total genomic DNA. To normalize the two channels with respect to overall intensity, we then adjusted photomultiplier and laser power settings such that the signal ratio at these elements was as close to 1.0 as possible. The combined images were analyzed with custom-written software. A bounding box, fitted to the size of the DNA spots in each quadrant, was placed over each array element. The average fluorescent intensity was calculated by summing the intensities of each pixel present in a bounding box, and then dividing by the total number of pixels. Local area background was calculated for each array element by determining the average fluorescent intensity for the lower 20% of pixel intensities. Although this method tends to underestimate the background, causing an underestimation of extreme ratios, it produces a very consistent and noise-tolerant approximation. Although the analog-to-digital board used for data collection possesses a wide dynamic range (12 bits), several signals were saturated (greater than the maximum signal intensity allowed) at the chosen settings. Therefore, extreme ratios at bright elements are generally underestimated. A signal was deemed significant if the average intensity after background subtraction was at least 2.5-fold higher than the standard deviation in the background measurements for all elements on the array.

50. In addition to the 17 genes shown in Table 1, three additional genes were induced by an average of more than threefold in the duplicate experiments, but in one of the two experiments, the induction was less than twofold (range 1.6- to 1.9-fold)

51. We thank H. Bennett, P. Spellman, J. Ravetto, M. Eisen, R. Pillai, B. Dunn, T. Ferea, and other members of the Brown lab for their assistance and helpful advice. We also thank S. Friend, D. Botstein, S. Smith, J. Hudson, and D. Dolginow for advice, support, and encouragement; K. Struhl and S. Chatterjee for the Tup1 deletion strain; L. Fernandes for helpful advice on Yap1; and S. Klapholz and the reviewers for many helpful comments on the manuscript. Supported by a grant from the National Human Genome Research Institute (NHGRI) (HG00450), and by the Howard Hughes Medical Institute (HHMI). J.D.R. was supported by the HHMI and the NHGRI. V.R. was supported in part by an Institutional Training Grant in Genome Science (T32 HG00044) from the NHGRI. P.O.B. is an associate investigator of the HHMI.

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FOCUS - 17 of 19 DOCUMENTS

August 11, 1997, Monday

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HEADLINE: Eli Lilly & Co. and **Acacia Biosciences** Enter Into Research Collaboration;
First Corporate Agreement for Acacia's Genome Reporter Matrix(TM)

DATELINE: RICHMOND, Calif., Aug. 11

BODY:

Acacia Biosciences and Eli Lilly and Company (Lilly) announced today the signing of a joint research collaboration to utilize Acacia's Genome Reporter Matrix(TM) (GRM) to aid in the selection and optimization of lead compounds. Under the collaboration, Acacia will provide chemical and biological profiles on a class of Lilly's compounds for an undisclosed fee.

Acacia's GRM is an assay-based computer modeling system that uses yeast as a miniature ecosystem. The GRM can profile the extent, nature and quantity of any changes in gene expression. Because of the similarities between the yeast and human genome, the system serves as an excellent surrogate for the human body, mimicking the effects induced by a biologically active molecule.

"Using yeast as a model organism for lead optimization makes a lot of sense given the high degree of homology with human metabolic pathways," said William Current of Lilly Research Laboratories. "Acacia's innovative GRM has the potential to provide enormous insight into the therapeutic impact of our compounds and make the drug discovery process more rational. It should substantially accelerate the development process."

"This first agreement with a major pharmaceutical company is an important milestone in the development of Acacia," said Bruce Cohen, President and CEO of Acacia. "The deal is in line with our strategy of establishing alliances that will allow our collaborators to use genomic profiles to identify and optimize compounds within their existing portfolios. In the long run, this technology can be used to characterize large scale combinatorial libraries, predict side effects prior to clinical trials and resurrect drugs that have failed during clinical trials."

The GRM incorporates two critical elements: chemical response profiles and genetic response profiles. The chemical response profiles measure the change in gene expression caused by potential therapeutics and then rank genes with altered expressions by degree of response. The genetic response profiles measure changes in gene expression caused by mutations in the genes encoding potential targets of pharmaceuticals; these genetic response profiles represent gold standards in drug discovery by defining the response profile expected for drugs with perfect selectivity and specificity. By comparing the two profiles, one can analyze a potential drug candidate's ability to mimic the action of a 'perfect' drug.

Acacia Biosciences is a functional genomics company developing proprietary technologies to enhance the speed and efficacy of drug discovery and development. Acacia's Genome Reporter Matrix capitalizes on the latest advances in genomics and combinatorial chemistry to generate comprehensive profiles of drug candidates' in vivo activity.
SOURCE Acacia Biosciences
CONTACT: Bruce Cohen, President and CEO of Acacia Biosciences, 510-669-2330 ext. 103 or Media: Linda Seaton of Feinstein

LOAD-DATE: August 12, 1997

Differential gene expression in drug metabolism and toxicology: practicalities, problems and potential

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1. An important feature of the work of many molecular biologists is identifying which genes are switched on and off in a cell under different environmental conditions or subsequent to xenobiotic challenge. Such information has many uses, including the deciphering of molecular pathways and facilitating the development of new experimental and diagnostic procedures. However, the student of gene hunting should be forgiven for perhaps becoming confused by the mountain of information available as there appears to be almost as many methods of discovering differentially expressed genes as there are research groups using the technique.

2. The aim of this review was to clarify the main methods of differential gene expression analysis and the mechanistic principles underlying them. Also included is a discussion on some of the practical aspects of using this technique. Emphasis is placed on the so-called 'open' systems, which require no prior knowledge of the genes contained within the study model. Whilst these will eventually be replaced by 'closed' systems in the study of human, mouse and other commonly studied laboratory animals, they will remain a powerful tool for those examining less fashionable models.

3. The use of suppression-PCR subtractive hybridization is exemplified in the identification of up- and down-regulated genes in rat liver following exposure to phenobarbital, a well-known inducer of the drug metabolizing enzymes.

4. Differential gene display provides a coherent platform for building libraries and microchip arrays of 'gene fingerprints' characteristic of known enzyme inducers and xenobiotic toxicants, which may be interrogated subsequently for the identification and characterization of xenobiotics of unknown biological properties.

Introduction

It is now apparent that the development of almost all cancers and many non-neoplastic diseases are accompanied by altered gene expression in the affected cells compared to their normal state (Hunter 1991, Wynford-Thomas 1991, Vogelstein and Kinzler 1993, Semenza 1994, Cassidy 1995, Kleinjan and Van Hegningen 1998). Such changes also occur in response to external stimuli such as pathogenic microorganisms (Rohn et al. 1996, Singh et al. 1997, Griffin and Krishna 1998, Lunney 1998) and xenobiotics (Sewall et al. 1995, Dogra et al. 1998, Ramana and Kohli 1998), as well as during the development of undifferentiated cells (Hecht 1998, Rudin and Thompson 1998, Schneider-Maunoury et al. 1998). The potential medical and therapeutic benefits of understanding the molecular changes which occur in any given cell in progressing from the normal to the 'altered' state are enormous. Such profiling essentially provides a 'fingerprint' of each step of a

cell's development or response and should help in the elucidation of specific and sensitive biomarkers representing, for example, different types of cancer or previous exposure to certain classes of chemicals that are enzyme inducers.

In drug metabolism, many of the xenobiotic-metabolizing enzymes (including the well-characterized isoforms of cytochrome P450) are inducible by drugs and chemicals in man (Pelkonen *et al.* 1998), predominantly involving transcriptional activation of not only the cognate cytochrome P450 genes, but additional cellular proteins which may be crucial to the phenomenon of induction. Accordingly, the development of methodology to identify and assess the full complement of genes that are either up- or down-regulated by inducers are crucial in the development of knowledge to understand the precise molecular mechanisms of enzyme induction and how this relates to drug action. Similarly, in the field of chemical-induced toxicity, it is now becoming increasingly obvious that most adverse reactions to drugs and chemicals are the result of multiple gene regulation, some of which are causal and some of which are casually-related to the toxicological phenomenon *per se*. This observation has led to an upsurge in interest in gene-profiling technologies which differentiate between the control and toxin-treated gene pools in target tissues and is, therefore, of value in rationalizing the molecular mechanisms of xenobiotic-induced toxicity. Knowledge of toxin-dependent gene regulation in target tissues is not solely an academic pursuit as much interest has been generated in the pharmaceutical industry to harness this technology in the early identification of toxic drug candidates, thereby shortening the developmental process and contributing substantially to the safety assessment of new drugs. For example, if the gene profile in response to say a testicular toxin that has been well-characterized *in vivo* could be determined in the testis, then this profile would be representative of all new drug candidates which act via this specific molecular mechanism of toxicity, thereby providing a useful and coherent approach to the early detection of such toxicants. Whereas it would be informative to know the identity and functionality of all genes up/down regulated by such toxicants, this would appear a longer term goal, as the majority of human genes have not yet been sequenced, far less their functionality determined. However, the current use of gene profiling yields a *pattern* of gene changes for a xenobiotic of unknown toxicity which may be matched to that of well-characterized toxins, thus alerting the toxicologist to possible *in vivo* similarities between the unknown and the standard, thereby providing a platform for more extensive toxicological examination. Such approaches are beginning to gain momentum, in that several biotechnology companies are commercially producing 'gene chips' or 'gene arrays' that may be interrogated for toxicity assessment of xenobiotics. These chips consist of hundreds/thousands of genes, some of which are degenerate in the sense that not all of the genes are mechanistically-related to any one toxicological phenomenon. Whereas these chips are useful in broad-spectrum screening, they are maturing at a substantial rate, in that gene arrays are now becoming more specific, e.g. chips for the identification of changes in growth factor families that contribute to the aetiology and development of chemically-induced neoplasias.

Although documenting and explaining these genetic changes presents a formidable obstacle to understanding the different mechanisms of development and disease progression, the technology is now available to begin attempting this difficult challenge. Indeed, several 'differential expression analysis' methods have been developed which facilitate the identification of gene products that demonstrate

altered expression in cells of one population compared to another. These methods have been used to identify differential gene expression in many situations, including invading pathogenic microbes (Zhao *et al.* 1998), in cells responding to extracellular and intracellular microbial invasion (Duguid and Dinauer 1990, Ragno *et al.* 1997, Maldarelli *et al.* 1998), in chemically treated cells (Syed *et al.* 1997, Rockett *et al.* 1999), neoplastic cells (Liang *et al.* 1992, Chang and Terzaghi-Howe 1998), activated cells (Gurskaya *et al.* 1996, Wan *et al.* 1996), differentiated cells (Hara *et al.* 1991, Guimaraes *et al.* 1995a, b), and different cell types (Davis *et al.* 1984, Hedrick *et al.* 1984, Xhu *et al.* 1998). Although differential expression analysis technologies are applicable to a broad range of models, perhaps their most important advantage is that, in most cases, absolutely no prior knowledge of the specific genes which are up- or down-regulated is required.

The field of differential expression analysis is a large and complex one, with many techniques available to the potential user. These can be categorized into several methodological approaches, including:

(1) Differential screening,
(2) Subtractive hybridization (SH) (includes methods such as chemical cross-linking subtraction—CCLS, suppression-PCR subtractive hybridization—SSH, and representational difference analysis—RDA),
(3) Differential display (DD),
(4) Restriction endonuclease facilitated analysis (including serial analysis of gene expression—SAGE—and gene expression fingerprinting—GEF),
(5) Gene expression arrays, and
(6) Expressed sequence tag (EST) analysis.

The above approaches have been used successfully to isolate differentially expressed genes in different model systems. However, each method has its own subtle (and sometimes not so subtle) characteristics which incur various advantages and disadvantages. Accordingly, it is the purpose of this review to clarify the mechanistic principles underlying the main differential expression methods and to highlight some of the broader considerations and implications of this very powerful and increasingly popular technique. Specifically, we will concentrate on the so-called 'open' systems, namely those which do not require any knowledge of gene sequences and, therefore, are useful for isolating unknown genes. Two 'closed' systems (those utilising previously identified gene sequences), EST analysis and the use of DNA arrays, will also be considered briefly for completeness. Whilst emphasis will often be placed on suppression PCR subtractive hybridization (SSH, the approach employed in this laboratory), it is the aim of the authors to highlight, wherever possible, those areas of common interest to those who use, or intend to use, differential gene expression analysis.

Differential cDNA library screening (DS)

Despite the development of multiple technological advances which have recently brought the field of gene expression profiling to the forefront of molecular analysis, recognition of the importance of differential gene expression and characterization of differentially expressed genes has existed for many years. One of the original approaches used to identify such genes was described 20 years ago by St John and Davis (1979). These authors developed a method, termed 'differential plaque filter

hybridization', which was used to isolate galactose-inducible DNA sequences from yeast. The theory is simple: a genomic DNA library is prepared from normal, unstimulated cells of the test organism/tissue and multiple filter replicas are prepared. These replica blots are probed with radioactively (or otherwise) labelled complex cDNA probes prepared from the control and test cell mRNA populations. Those mRNAs which are differentially expressed in the treated cell population will show a positive signal only on the filter probed with cDNA from the treated cells. Furthermore, labelled cDNA from different test conditions can be used to probe multiple blots, thereby enabling the identification of mRNAs which are only up-regulated under certain conditions. For example, St John and Davis (1979) screened replica filters with acetate-, glucose- and galactose-derived probes in order to obtain genes induced specifically by galactose metabolism. Although groundbreaking in its time this method is now considered insensitive and time-consuming, as up to 2 months are required to complete the identification of genes which are differentially expressed in the test population. In addition, there is no convenient way to check that the procedure has worked until the whole process has been completed.

Subtractive Hybridization (SH)

The developing concept of differential gene expression and the success of early approaches such as that described by St John and Davis (1979) soon gave rise to a search for more convenient methods of analysis. One of the first to be developed was SH, numerous variations of which have since been reported (see below). In general, this approach involves hybridization of mRNA/cDNA from one population (tester) to excess mRNA/cDNA from another (driver), followed by separation of the unhybridized tester fraction (differentially expressed) from the hybridized common sequences. This step has been achieved physically, chemically and through the use of selective polymerase chain reaction (PCR) techniques.

Physical separation

Original subtractive hybridization technology involved the physical separation of hybridized common species from unique single stranded species. Several methods of achieving this have been described, including hydroxyapatite chromatography (Sargent and Dawid 1983), avidin-biotin technology (Duguid and Dinauer 1990) and oligodT-latex separation (Hara *et al.* 1991). In the first approach, common mRNA species are removed by cDNA (from test cells)-mRNA (from control cells) subtractive hybridization followed by hydroxyapatite chromatography, as hydroxy-apatite specifically adsorbs the cDNA-mRNA hybrids. The unabsorbed cDNA is then used either for the construction of a cDNA library of differentially expressed genes (Sargent and Dawid 1983, Schneider *et al.* 1988) or directly as a probe to screen a preselected library (Zimmerman *et al.* 1980, Davis *et al.* 1984, Hedrick *et al.* 1984). A schematic diagram of the procedure is shown in figure 1.

Less rigorous physical separation procedures coupled with sensitivity enhancing PCR steps were later developed as a means to overcome some of the problems encountered with the hydroxyapatite procedure. For example, Daguid and Dinauer (1990) described a method of subtraction utilizing biotin-affinity systems as a means to remove hybridized common sequences. In this process, both the control and tester mRNA populations are first converted to cDNA and an adaptor ('oligovector',

Control (driver) mRNA   Tester (test) cDNA (1st strand)

```
————AAAA                    ————TTTTT
————AAAA                    ————TTTTT
————AAAA                    ————TTTTT
————AAAA                    ————TTTTT
```

Mix (ratio >35:1) & hybridize

```
————AAAA        ————AAAA
————TTTT        ————AAAA
————AAAA
————TTTT        ————TTTT

————TTTT
```

Hydroxyapatite chromatography ——→ RNA:cDNA hybrids removed

Unhybridized
cDNA (differentially expressed)
and mRNA

```
————TTTT
————TTTTT
————AAAA

————AAAA
```

Sepharose CL6B exclusion ——→ Small cDNA fragments (<450bp)
chromatography

Enriched, differentially expressed cDNA

or

Produce clones   Label directly and probe library

Figure 1. The hydroxyapatite method of subtractive hybridization. cDNA derived from the treated/altered (tester) population is mixed with a large excess of mRNA from the control (driver) population. Following hybridization, mRNA-cDNA hybrids are removed by hydroxyapatite chromatography. The only cDNAs which remain are those which are differentially expressed in the treated/altered population. In order to facilitate the recovery of full length clones, small cDNA fragments are removed by exclusion chromatography. The remaining cDNAs are then cloned into a vector for sequencing, or labelled and used directly to probe a library, as described by Sargent and Dawid (1983).

containing a restriction site) ligated to both sides. Both populations are then amplified by PCR, but the driver cDNA population is subsequently digested with the adaptor-containing restriction endonuclease. This serves to cleave the oligo-vector and reduce the amplification potential of the control population. The digested control population is then biotinylated and an excess mixed with tester cDNA. Following denaturation and hybridization, the mix is applied to a biocytin column (streptavidin may also be used) to remove the control population, including heteroduplexes formed by annealing of common sequences from the tester population. The procedure is repeated several times following the addition of fresh

Control (driver) mRNA

Test (tester) mRNA

Anneal mRNA to polydT₃₀ latex beads

cDNA synthesis

Mix and anneal

Centrifuge beads, collect and store supernatant,
dissociate polyA, reapply supernatant

Tester-specific mRNA retrieved after
4 rounds of hybridization

cDNA synthesis

Ligate adaptors and insert into vector

Sequence inserts and/or carry out
other downstream applications

Figure 2. The use of oligodT₃₀ latex to perform subtractive hybridization. mRNA extracted from the
control (driver) population is converted to anchored cDNA using polydT oligonucleotides
attached to latex beads. mRNA from the treated/altered (tester) population is repeatedly
hybridized against an excess of the anchored driver cDNA. The final population of mRNA is
tester specific and can be converted into cDNA for cloning and other downstream applications, as
described by Hara *et al.* (1991).

control cDNA. In order to further enrich those species differentially expressed in the tester cDNA, the subtracted tester population is amplified by PCR following every second subtraction cycle. After six cycles of subtraction (three reamplification steps) the reaction mix is ligated into a vector for further analysis.

In a slightly different approach, Hara *et al.* (1991) utilized a method whereby oligo(dT$_{30}$) primers attached to a latex substrate are used to first capture mRNA extracted from the control population. Following 1st strand cDNA synthesis, the RNA strand of the heteroduplexes is removed by heat denaturation and centrifugation (the cDNA-oligotex-dT$_{30}$ forms a pellet and the supernatant is removed). A quantity of tester mRNA is then repeatedly hybridized to the immobilized control (driver) cDNA (which is present in 20-fold excess). After several rounds of hybridization the only mRNA molecules left in the tester mRNA population are those which are not found in the driver cDNA-oligotex-dT$_{30}$ population. These tester-specific mRNA species are then converted to cDNA and, following the addition of adaptor sequences, amplified by PCR. The PCR products are then ligated into a vector for further analysis using restriction sites incorporated into the PCR primers. A schematic illustration of this subtraction process is shown in figure 2.

However, all these methods utilising physical separation have been described as inefficient due to the requirement for large starting amounts of mRNA, significant loss of material during the separation process and a need for several rounds of hybridization. Hence, new methods of differential expression analysis have recently been designed to eliminate these problems.

Chemical Cross-Linking Subtraction (CCLS)

In this technique, originally described by Hampson *et al.* (1992), driver mRNA is mixed with tester cDNA (1st strand only) in a ratio of > 20:1. The common sequences form cDNA:mRNA hybrids, leaving the tester specific species as single stranded cDNA. Instead of physically separating these hybrids, they are inactivated chemically using 2,5 diaziridinyl-1,4-benzoquinone (DZQ). Labelled probes are then synthesized from the remaining single stranded cDNA species (unreacted mRNA species remaining from the driver are not converted into probe material due to specificity of Sequenase T7 DNA polymerase used to make the probe) and used to screen a cDNA library made from the tester cell population. A schematic diagram of the system is shown in figure 3.

It has been shown that the differentially expressed sequences can be enriched at least 300-fold with one round of subtraction (Hampson *et al.* 1992), and that the technique should allow isolation of cDNAs derived from transcripts that are present at less than 50 copies per cell. This equates to genes at the low end of intermediate abundance (see table 1). The main advantages of the CCLS approach are that it is rapid, technically simple and also produces fewer false positives than other differential expression analysis methods. However, like the physical separation protocols, a major drawback with CCLS is the large amount of starting material required (at least 10 μg RNA). Consequently, the technique has recently been refined so that a renewable source of RNA can be generated. The degenerate random oligonucleotide primed (DROP) adaptation (Hampson *et al.* 1996, Hampson and Hampson 1997) uses random hexanucleotide sequences to prime solid phase-synthesized cDNA. Since each primer includes a T7 polymerase promotor sequence

Control (driver) mRNA   Test (tester) mRNA

————AAAA   ···············AAAA

————AAAA   ···············AAAA

1st strand cDNA synthesis
followed by alkaline hydrolysis ↓

————TTTT
————TTTT
————TTTT

Mix and anneal

↓

mRNA:cDNA hybrids   ————AAAA
                   ————TTTT

+

Unique cDNA species   ————TTTT

↓   Cross linking agent
      (DZQ) added

Hybrids are cross-linked   xxxxxxxxx————AAAA
                    ————TTTT

+

————TTTT

↓

Probes synthesised from single stranded cDNA
species and used to probe cDNA library
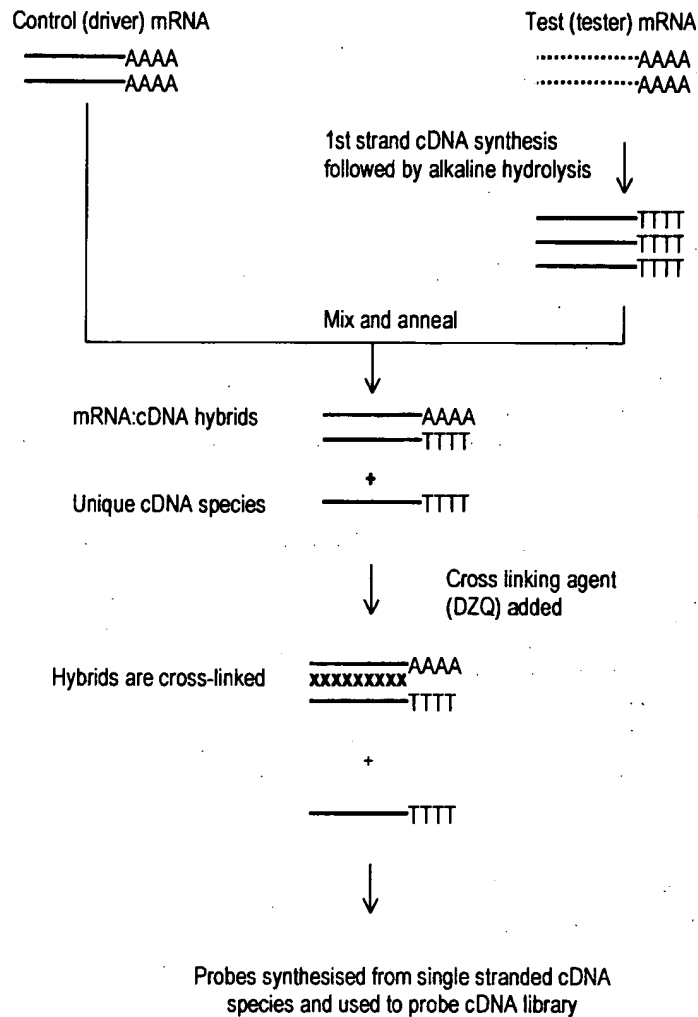
Figure 3. Chemical cross-linking subtraction. Excess driver mRNA is mixed with 1st strand tester cDNA. The common sequences form mRNA:cDNA hybrids which are cross linked with 2,5 diaziridinyl-1,4-benzoquinone (DZQ) and the remaining cDNA sequences are differentially expressed in the tester population. Probes are made from these sequences using Sequenase 2.0 DNA polymerase, which lacks reverse transcriptase activity and, therefore, does not react with the remaining mRNA molecules from the driver. The labelled probes are then used to screen a cDNA library for clones of differentially expressed sequences. Adapted from Walter *et al.* (1996), with permission.

Table 1. The abundance of mRNA species and classes in a typical mammalian cell.

mRNA class	Copies of each species/cell	No. of mRNA species in class	Mean % of each species in class	Mean mass (ng) of each species/μg total RNA
Abundant	12000	4	3.3	1.65
Intermediate	300	500	0.08	0.04
Rare	15	11000	0.004	0.002

Modified from Bertioli *et al.* (1995).

at the 5´end, the final pool of random cDNA fragments is a PCR-renewable cDNA population which is representative of the expressed gene pool and can be used to synthesize sense RNA for use as driver material. Furthermore, if the final pool of random cDNA fragments is reamplified using biotinylated T7 primer and random hexamer, the product can be captured with streptavidin beads and the antisense strand eluted for use as tester. Since both target and driver can be generated from the same DROP product, subtraction can be performed in both directions (i.e. for up- and down-regulated species) between two different DROP products.

Representational Difference Analysis (RDA)

RDA of cDNA (Hubank and Schatz 1994) is an extension of the technique originally applied to genomic DNA as a means of identifying differences between two complex genomes (Lisitsyn *et al.* 1993). It is a process of subtraction and amplification involving subtractive hybridization of the tester in the presence of excess driver. Sequences in the tester that have homologues in the driver are rendered unamplifiable, whereas those genes expressed only in the tester retain the ability to be amplified by PCR. The procedure is shown schematically in figure 4.

In essence, the driver and tester mRNA populations are first converted to cDNA and amplified by PCR following the ligation of an adaptor. The adaptors are then removed from both populations and a new (different) adaptor ligated to the amplified tester population only. Driver and tester populations are next melted and hybridized together in a ratio of 100:1. Following hybridization, only tester:tester homohybrids have 5´adaptors at each end of the DNA duplex and can, thus, be filled in at both 3´ends. Hence, only these molecules are amplified exponentially during the subsequent PCR step. Although tester:driver heterohybrids are present, they only amplify in a linear fashion, since the strand derived from the driver has no adaptor to which the primer can bind. Driver:driver heterohybrids have no adaptors and, therefore, are not amplified. Single stranded molecules are digested with mung bean nuclease before a further PCR-enrichment of the tester:tester homohybrids. The adaptors on the amplified tester population are then replaced and the whole process repeated a further two or three times using an increasing excess of driver (Hubank and Shatz used a tester:driver ratio of 1:400, 1:80000 and 1:800000 for the second, third and fourth hybridizations, respectively). Different adaptors are ligated to the tester between successive rounds of hybridization and amplification to prevent the accumulation of PCR products that might interfere with subsequent amplifications. The final display is a series of differentially expressed gene products easily observable on an ethidium bromide gel.

The main advantages of RDA are that it offers a reproducible and sensitive approach to the analysis of differentially expressed genes. Hubank and Schatz (1994) reported that they were able to isolate genes that were differentially expressed in substantially less than 1% of the cells from which the tester is derived. Perhaps the main drawback is that multiple rounds of ligation, hybridization, amplifiation and digestion are required. The procedure is, therefore, lengthier than many other differential display approaches and provides more opportunity for operator-induced error to occur. Although the generation of false positives has been noted, this has been solved to some degree by O'Neill and Sinclair (1997) through the use of HPLC-purified adaptors. These are free of the truncated adaptors which appear to be a major source of the false positive bands. A very similar technique to RDA, termed linker capture subtraction (LCS) was described by Yang and Sytowski (1996).

ds control (driver) cDNA ds test (tester) cDNA

Digest with restriction enzyme

Ligate to
dephosphorylated
12/24 adaptor
strands

Melt 12mer

Fill in 3' ends (Taq), add
primer (———) and
amplify

Digest Digest and ligate
new 12/24 adaptor

Mix 100:1, melt and hybridize

Fill in ends, add primer (——) and amplify

Linear amplification   Exponential amplification   No amplification

Digest PCR products with mung bean nuclease to remove
ssDNA molecules present after amplification

First difference

Figure 4. The representational difference analysis (RDA) technique. Driver and tester cDNA are digested with a 4-cutter restriction enzyme such as *Dpn*II. The 1st set of 12/24 adaptor strands (oligonucleotides) are ligated to each other and the digested cDNA products. The 12mer is subsequently melted away and the 3'ends filled in using Taq DNA polymerase. Each cDNA population is then amplified using PCR, following which the 1st set of adaptors is removed with *Dpn*II. A second set of 12/24 adaptor strands is then added to the amplified tester cDNA population, after which the tester is hybridized against a large excess of driver. The 12mer adaptors are melted and the 3'ends filled in as before. PCR is carried out with primers identical to the new 24mer adaptor. Thus, the only hybridization products which are exponentially amplified are those which are tester:tester combinations. Following PCR, ssDNA products are removed with mung bean nuclease, leaving the 'first difference product'. This is digested and a third set of 12/24 adaptors added before repeating the subtraction process from the hybridization stage. The process is repeated to the 3rd or 4th difference product, as described by Lisitsyn *et al.* (1993) and Hubank and Schatz (1994).

Suppression PCR Subtractive Hybridization (SSH)

The most recent adaptation of the SH approach to differential expression analysis was first described by Diatchenko *et al.* (1996) and Gurskaya *et al.* (1996). They reported that a 1000–5000 fold enrichment of rare cDNAs (equivalent to isolating mRNAs present at only a few copies per cell) can be obtained without the need for multiple hybridizations/subtractions. Instead of physical or chemical removal of the common sequences, a PCR-based suppression system is used (see figure 5).

In SSH, excess driver cDNA is added to two portions of the tester cDNA which have been ligated with different adaptors. A first round of hybridization serves to enrich differentially expressed genes and equalize rare and abundant messages. Equalization occurs since reannealing is more rapid for abundant molecules than for rarer molecules due to the second order kinetics of hybridization (James and Higgins 1985). The two primary hybridization mixes are then mixed together in the presence of excess driver and allowed to hybridize further. This step permits the annealing of single stranded complementary sequences which did not hybridize in the primary hybridization, and in doing so generates templates for PCR amplification. Although there are several possible combinations of the single stranded molecules present in the secondary hybridization mix, only one particular combination (differentially expressed in the tester cDNA composed of complimentary strands having different adaptors) can amplify exponentially.

Having obtained the final differential display, two options are available if cloning of cDNAs is desired. One is to transform the whole of the final PCR reaction into competent cells. Transformed colonies can then be isolated and their inserts characterized by sequencing, restriction analysis or PCR. Alternatively, the final PCR products can be resolved on a gel and the individual bands excised, reamplified and cloned. The first approach is technically simpler and less time consuming. However, ligation/transformation reactions are known to be biased towards the cloning of smaller molecules, and so the final population of clones will probably not contain a representative selection of the larger products. In addition, although equalization theoretically occurs, observations in this laboratory suggest that this is by no means perfectly accomplished. Consequently, some gene species are present in a higher number than others and this will be represented in the final population of clones. Thus, in order to obtain a substantial proportion of those gene species that actually demonstrate differential expression in the tester population, the number of clones that will have to be screened after this step may be substantial. The second approach is initially more time consuming and technically demanding. However, it would appear to offer better prospects for cloning larger and low abundance gel products. In addition, one can incorporate a screening step that differentiates different products of different sequences but of the same size (HA-staining, see later). In this way, a good idea of the final number of clones to be isolated and identified can be achieved.

An alternative (or even complementary) approach is to use the final differential display reaction to screen a cDNA library to isolate full length clones for further characterization, or a DNA array (see later) to quickly identify known genes. SSH has been used in this laboratory to begin characterization of the short-term gene expression profiles of enzyme-inducers such as phenobarbital (Rockett *et al.* 1997) and Wy-14,643 (Rockett *et al.* unpublished observations). The isolation of differentially expressed genes in this manner enables the construction of a fingerprint

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Tester cDNA with adaptor 1

Driver cDNA (in excess)

Tester cDNA with adaptor 2

First Hybridization

Mix samples, add fresh denatured driver, anneal

a, b, c, d & e

Fill in ends

a

b

c

d

e

Add primers and amplify by PCR

a, d no amplification

b no amplification - suppressed due to formation of panhandle structure

c linear amplification

e exponential amplification
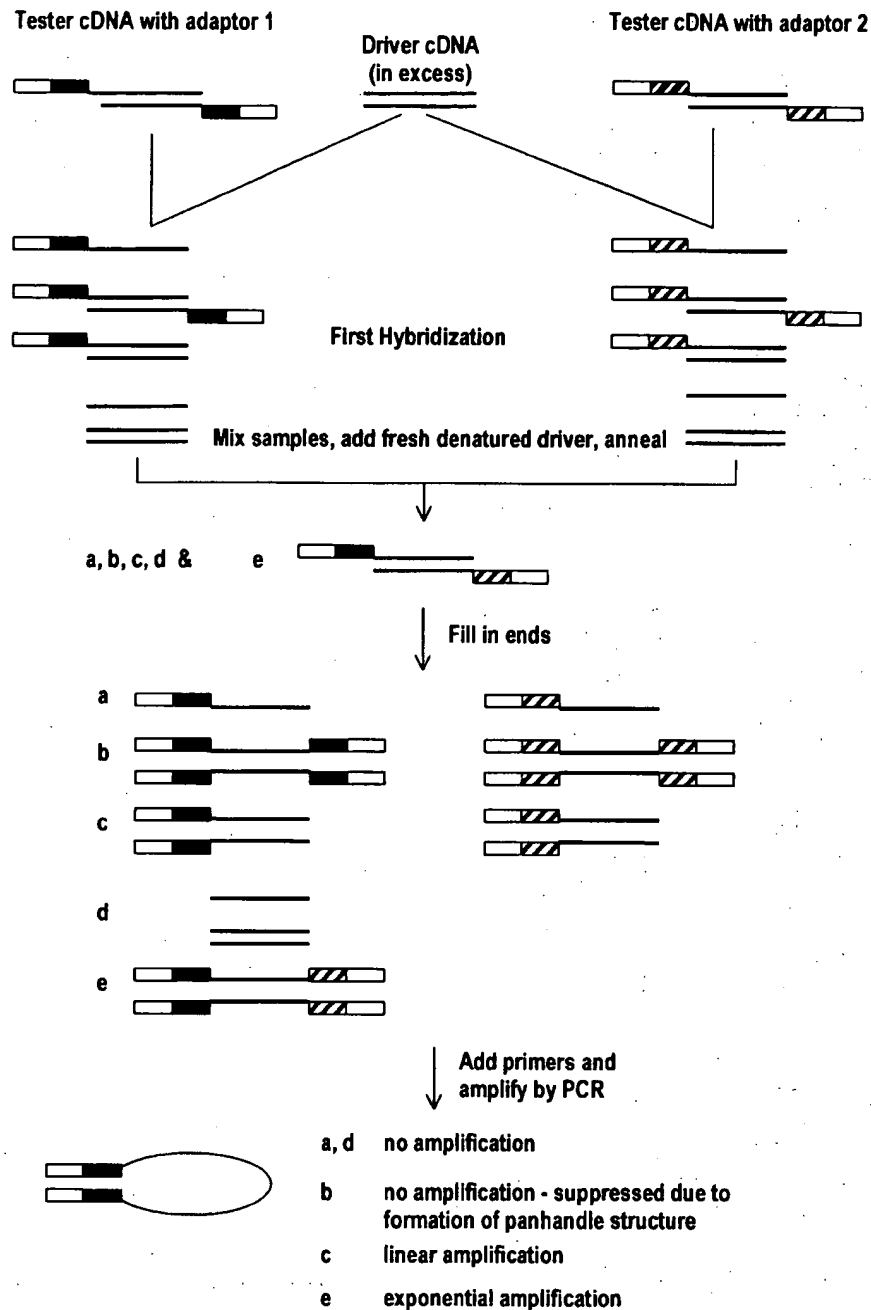
Figure 5. PCR-select cDNA subtraction. In the primary hybridization, an excess of driver cDNA is added to each tester cDNA population. The samples are heat denatured and allowed to hybridize for between 3 and 8 h. This serves two purposes: (1) to equalize rare and abundant molecules; and (2) to enrich for differentially expressed sequences—cDNAs that are not differentially expressed form type c molecules with the driver. In the secondary hybridization, the two primary hybridizations are mixed together without denaturing. Fresh denatured driver can also be added at this point to allow further enrichment of differentially expressed sequences. Type e molecules are formed in this secondary hybridization which are subsequently amplified using two rounds of PCR. The final products can be visualized on an agarose gel, labelled directly or cloned into a vector for downstream manipulation. As described by Diatchenko *et al.* (1996) and Gurskaya *et al.* (1996), with permission.
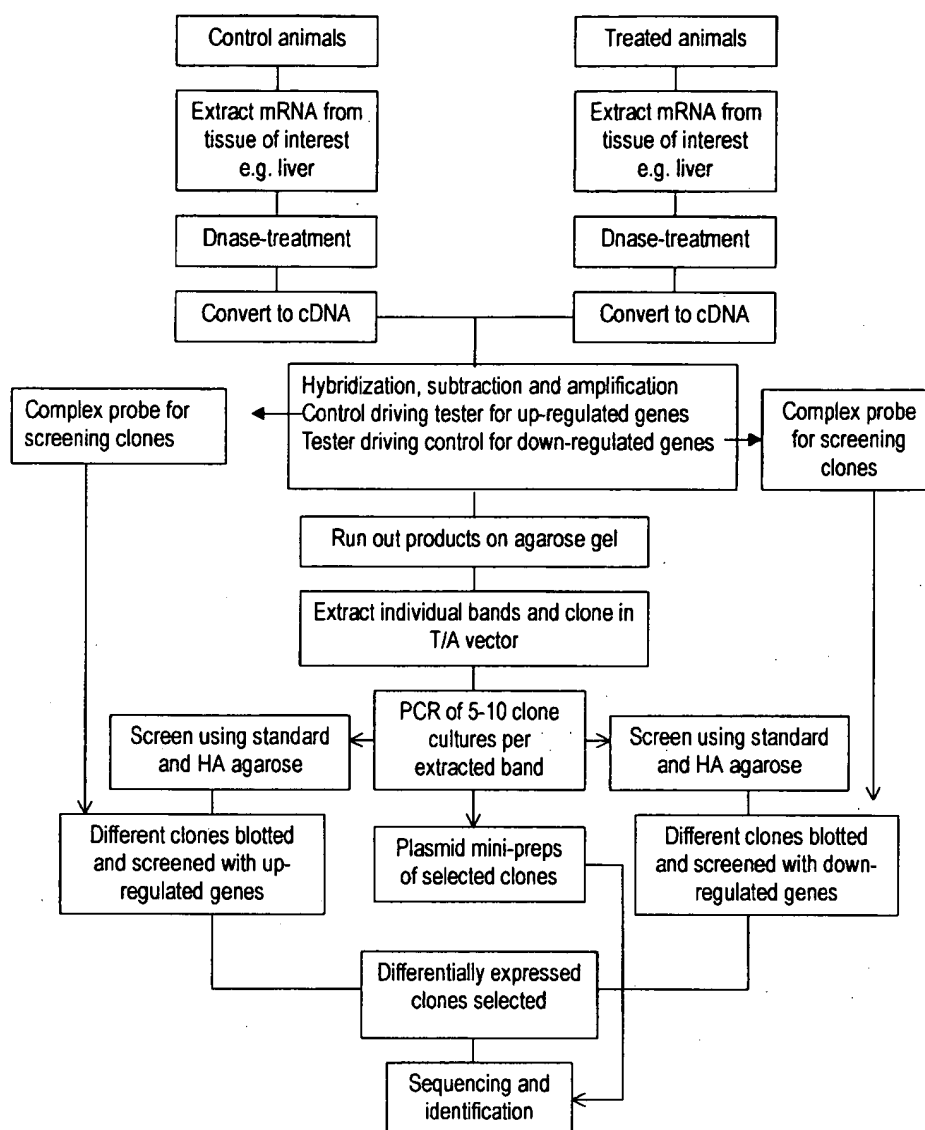
Figure 6. Flow diagram showing method used in this laboratory to isolate and identify clones of genes which are differentially expressed in rat liver following short term exposure to the enzyme inducers, phenobarbital and Wy-14,643.

of expressed genes which are unique to each compound and time/dose point. Such information could be useful in short-term characterization of the toxic potential of new compounds by comparing the gene-expression profiles they elicit with those produced by known inducers. Figure 6 shows a flow diagram of the method used to isolate, verify and clone differentially expressed genes, and figure 7 shows expression profiles obtained from a typical SSH experiment. Subsequent sub-cloning of the individual bands, sequencing and gene data base interrogation reveals many genes which are either up- or down-regulated by phenobarbital in the rat (tables 2 and 3).

One of the advantages in using the SSH approach is that no prior knowledge is required of which specific genes are up/down-regulated subsequent to xenobiotic
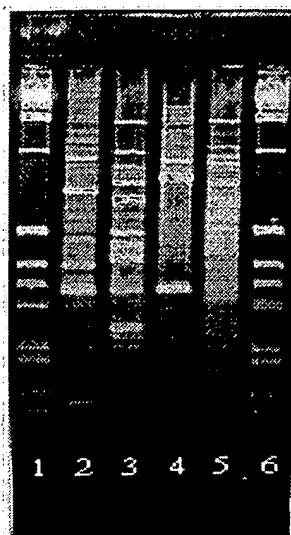
Figure 7. SSH display patterns obtained from rat liver following 3-day treatment with WY-14,643 or phenobarbital. mRNA extracted from control and treated livers was used to generate the differential displays using the PCR-Select cDNA subtraction kit (Clontech). Lane: 1—1kb ladder; 2—genes upregulated following Wy,14–643 treatment; 3—genes downregulated following Wy,14–643 treatment; 4—genes upregulated following phenobarbital treatment; 5—genes downregulated following phenobarbital treatment; 6—1kb ladder. Reproduced from Rockett *et al.* (1997), with permission.

exposure, and an almost complete complement of genes are obtained. For example, the peroxisome proliferator and non-genotoxic hepatocarcinogen Wy,14,643, up-regulates at least 28 genes and down-regulates at least 15 in the rat (a sensitive species) and produces 48 up- and 37 down-regulated genes in the guinea pig, a resistant species (Rockett, Swales, Esda and Gibson, unpublished observations). One of these genes, CD81, was up-regulated in the rat and down-regulated in the guinea pig following Wy-14,643 treatment. CD81 (alternatively named TAPA-1) is a widely expressed cell surface protein which is involved in a large number of cellular processes including adhesion, activation, proliferation and differentiation (Levy *et al.* 1998). Since all of these functions are altered to some extent in the phenomena of hepatomegaly and non-genotoxic hepatocarcinogenesis, it is intriguing, and probably mechanistically-relevant, that CD81 expression is differentially regulated in a resistant and susceptible species. However, the down-side of this approach is that the majority of genes can be sequenced and matched to database sequences, but the latter are predominantly expressed sequence tags or genes of completely unknown function, thus partially obscuring a realistic overall assessment of the critical genes of genuine biological interest. Notwithstanding the lack of complete funtional identification of altered gene expression, such gene profiling studies essentially provides a 'molecular fingerprint' in response to xenobiotic challenge, thereby serving as a mechanistically-relevant platform for further detailed investigations.

Differential Display (DD)

Originally described as 'RNA fingerprinting by arbitrarily primed PCR' (Liang and Pardee 1992) this method is now more commonly referred to as 'differential

Table 2.   Genes up-regulated in rat liver following 3-day exposure to phenobarbital.

Band number (approximate size in bp)	Highest sequence similarity		FASTA-EMBL gene identification
5 (1300)		93.5%	CYP2B1
7 (1000)		95.1%	Preproalbumin Serum albumin mRNA
8 (950)		98.3%	NC1-CGAP-Pr1 *H. sapiens* (EST)
10 (850)		95.7%	CYP2B1
11 (800)	Clone 1	94.9%	CYP2B1
	Clone 2	75.3%	CYP2B2
12 (750)		93.8%	TRPM-2 mRNA Sulfated glycoprotein
15 (600)		92.9%	Preproalbumin Serum albumin mRNA
16 (55)	Clone 1	95.2%	CYP2B1
	Clone 2	93.6%	Haptoglobulin mRNA partial alpha
21 (350)		99.3%	18S, 5.8S & 28S rRNa

Bands 1–4, 6, 9, 13, 14, and 17–20 are shown to be false positives by dot blot anaylsis and, therefore, are not sequenced. Derived from Rockett *et al.* (1997). It should be noted that the above genes do not represent the complete spectrum of genes which are up-regulated in rat liver by phenobarbital, but simply represents the genes sequenced and identified to date.

Table 3.   Genes down-regulated in rat liver following 3-day exposure to phenobarbital.

Band number (approximate size in bp)	Highest sequence similarity		FASTA-EMBL gene identification
1 (1500)		95.3%	3-oxoacyl-CoA thiolase
2 (1200)		92.3%	Hemopoxin mRNA
3 (1000)		91.7%	Alpha-2u-globulin mRNA
7 (700)	Clone 1	77.2%	*M.musculus* C1 inhibitor
	Clone 2	94.5%	Electron transfer flavoprotein
	Clone 3	91.0%	*M. musculus* Topoisomerase 1 (Topo 1)
8 (650)	Clone 1	86.9%	Soares 2NbMT *M. musculus* (EST)
	Clone 2	96.2%	Alpha-2u-globulin (s-type) mRNA
9 (600)	Clone 1	86.9%	Soares mouse NML *M. musculus* (EST)
	Clone 2	82.0%	Soares p3NMF 19.5 *M. musculus* (EST)
10 (550)		73.8%	Soares mouse NML *M. musculus* (EST)
11 (525)		95.7%	NC1-CGAP-Pr1 *H. sapiens* (EST)
12 (375)		100.0%	Ribosomal protein
13 (23)	Clone 1	97.2%	Soares mouse embryo NbME135 (EST)
	Clone 2	100.0%	Fibrinogen B-beta-chain
	Clone 3	100.0%	Apolipoprotein E gene
14 (170)		96.0%	Soares p3NMF19.5 *M. musculus* (EST)
15 (140)		97.3%	Stratagene mouse testis (EST)
Others: (300)		96.7%	*R. norvegicus* RASP 1 mRNA
(275)		93.1%	Soares mouse mammary gland (EST)

EST = Expressed sequence tag. Bands 4–6 were shown to be false positives by dot-blot analysis and, therefore, were not sequenced. Derived from Rockett *et al.* (1997). It should be noted that the above genes do not represent the complete spectrum of genes which are down-regulated in rat liver by phenobarbital, but simiply represents the genes sequenced and identified to date.

display' (DD). In this method, all the mRNA species in the control and treated cell populations are amplified in separate reactions using reverse transcriptase-PCR (RT-PCR). The products are then run side-by-side on sequencing gels. Those bands which are present in one display only, or which are much more intense in one

display compared to the other, are differentially expressed and may be recovered for further characterization. One advantage of this system is the speed with which it can be carried out—2 days to obtain a display and as little as a week to make and identify clones.

Two commonly used variations are based on different methods of priming the reverse transcription step (figure 8). One is to use an oligo dT with a 2-base 'anchor' at the 3'-end, e.g. 5′ (dT$_{11}$)CA 3′ (Liang and Pardee 1992). Alternatively, an arbitrary primer may be used for 1st strand cDNA synthesis (Welsh et al. 1992). This variant of RNA fingerprinting has also been called 'RAP' (RNA Arbitrarily Primed)-PCR. One advantage of this second approach is that PCR products may be derived from anywhere in the RNA, including open reading frames. In addition, it can be used for mRNAs that are not polyadenylated, such as many bacterial mRNAs (Wong and McClelland 1994). In both cases, following reverse transcription and denaturation, second strand cDNA synthesis is carried out with an arbitrary primer (*arbitrary* primers have a single base at each position, as compared to *random* primers, which contain a mixture of all four bases at each position). The resulting PCR, thus, produces a series of products which, depending on the system (primer · length and composition, polymerase and gel system), usually includes 50–100 products per primer set (Band and Sager 1989). When a combination of different dT-anchors and arbitrary primers are used, almost all mRNA species from a cell can be amplified. When the cDNA products from two different populations are analysed side by side on a polyacrylamide gel, differences in expression can be identified and the appropriate bands recovered for cloning and further analysis.

Although DD is perhaps the most popular approach used today for identifying differentially expressed genes, it does suffer from several perceived disadvantages:

(1) It may have a strong bias towards high copy number mRNAs (Bertioli et al. 1995), although this has been disputed (Wan et al. 1996) and the isolation of very low abundance genes may be achieved in certain circumstances (Guimeraes et al. 1995a).

(2) The cDNAs obtained often only represent the extreme 3′ end of the mRNA (often the 3′-untranslated region), although this may not always be the case (Guimeraes et al. 1995a). Since the 3′ end is often not included in Genbank and shows variation between organisms, cDNAs identified by DD cannot always be matched with their genes, even if they have been identified.

(3) The pattern of differential expression seen on the display often cannot be reproduced on Northern blots, with false positives arising in up to 70% of cases (Sun et al. 1994). Some adaptations have been shown to reduce false positives, including the use of two reverse transcriptases (Sung and Denman 1997), comparison of uninduced and induced cells over a time course (Burn et al. 1994) and comparison of DDPCR-products from two uninduced and two induced lines (Sompayrac et al. 1995). The latter authors also reported that the use of cytoplasmic RNA rather then total RNA reduces false positives arising from nuclear RNA that is not transported to the cytoplasm.

Further details of the background, strengths and weaknesses of the DD technique can be obtained from a review by McClelland et al. (1996) and from articles by Liang et al. (1995) and Wan et al. (1996).

mRNA
————————————AAAAAAAA

(dT₁₁)CA: AC ————

Arbitrary primer: ——

1ˢᵗ strand cDNA

←——————— AC ————

————————————UGAAAAAAA

1ˢᵗ strand cDNA

←————

————————————AAAAAAA

Denature and synthesise 2ⁿᵈ strand
with any arbitrary primer (———)

2ⁿᵈ strand cDNA

————————————→

←————— AC ————

2ⁿᵈ strand cDNA

————————————→

←————

————————————→

————————————→

cDNA can now be amplified by PCR using original primer pair
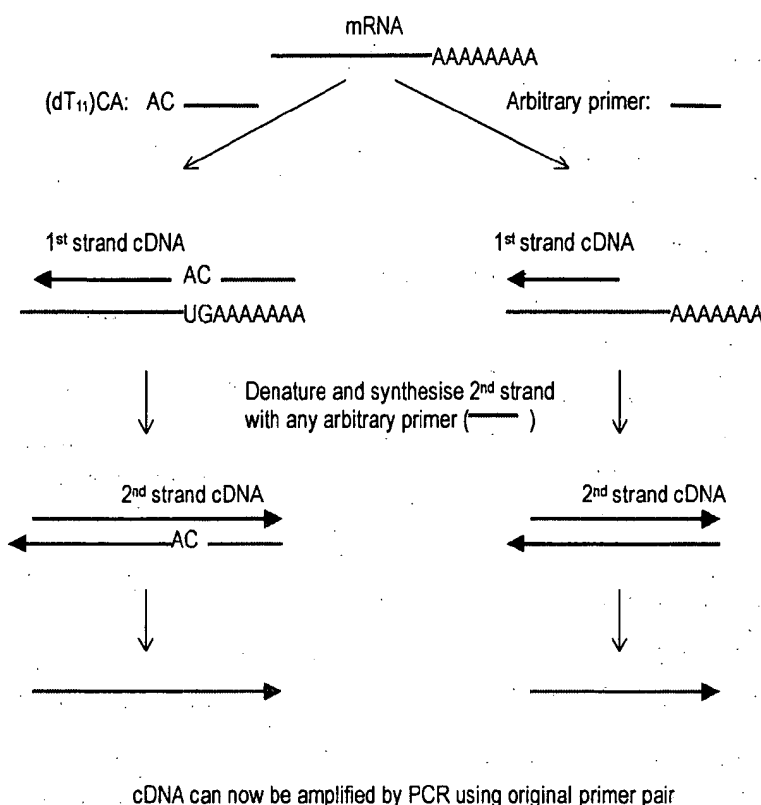
Figure 8. Two approaches to differential display (DD) analysis. 1ˢᵗ strand synthesis can be carried out
either with a polydT₁₁NN primer (where N = G, C or A) or with an arbitrary primer. The use of
different combinations of G, C and A to anchor the first strand polydT primer enables the priming
of the majority of polyadenylated mRNAs. Arbitrary primers may hybridize at none, one or more
places along the length of the mRNA, allowing 1ˢᵗ strand cDNA synthesis to occur at none, one
or more points in the same gene. In both cases, 2ⁿᵈ strand synthesis is carried out with an arbitrary
primer. Since these arbitrary primers for the 2ⁿᵈ strand may also hybridize to the 1ˢᵗ strand cDNA
in a number of different places, several different 2ⁿᵈ strand products may be obtained from one
binding point of the 1ˢᵗ strand primer. Following 2ⁿᵈ strand synthesis, the original set of primers
is used to amplify the second strand products, with the result that numerous gene sequences are
amplified.

Restriction endonuclease-facilitated analysis of gene expression

Serial Analysis of Gene Expression (SAGE)

A more recent development in the field of differential display is SAGE analysis
(Velculescu *et al.* 1995). This method uses a different approach to those discussed so
far and is based on two principles. Firstly, in more than 95% of cases, short
nucleotide sequences ('tags') of only nine or 10 base pairs provide sufficient
information to identify their gene of origin. Secondly, concatonation (linking
together in a series) of these tags allows sequencing of multiple cDNAs within a
single clone. Figure 9 shows a schematic representation of the SAGE process. In this
procedure, double stranded cDNA from the test cells is synthesized with a
biotinylated polydT primer. Following digestion with a commonly cutting (4bp
recognition sequence) restriction enzyme ('anchoring enzyme'), the 3′ ends of the
cDNA population are captured with streptavidin beads. The captured population is

split into two and different adaptors ligated to the 5´ends of each group. Incorporated into the adaptors is a recognition sequence for a type IIS restriction enzyme—one which cuts DNA at a defined distance (< 20 bp) from its recognition sequence. Hence, following digestion of each captured cDNA population with the IIS enzyme, the adaptors plus a short piece of the captured cDNA are released. The two populations are then ligated and the products amplified. The amplified products are cleaved with the original anchoring enzyme, religated (concatomers are formed in the process) and cloned. The advantage of this system is that hundreds of gene tags can be identified by sequencing only a few clones. Furthermore, the number of times a given transcript is identified is a quantitative measurement of that gene's abundance in the original population, a feature which facilitates identification of differentially expressed genes in different cell populations.

Some disadvantages of SAGE analysis include the technical difficulty of the method, a large amount of accurate sequencing is required, biased towards abundant mRNAs, has not been validated in the pharmaco/toxicogenomic setting and has only been used to examine well known tissue differences to date.

Gene Expression Fingerprinting (GEF)

A different capture/restriction digest approach for isolating differentially expressed genes has been described by Ivanova and Belyavsky (1995). In this method, RNA is converted to cDNA using biotinylated oligo(dT) primers. The cDNA population is then digested with a specific endonuclease and captured with magnetic streptavidin microbeads to facilitate removal of the unwanted 5´ digestion products. The use of restricted 3´-ends alone serves to reduce the complexity of the cDNA fragment pool and helps to ensure that each RNA species is represented by not more than one restriction product. An adaptor is ligated to facilitate subsequent amplification of the captured population. PCR is carried out with one adaptor-specific and one biotinylated polydT primer. The reamplified population is recaptured and the non-biotinylated strands removed by alkaline dissociation. The non-biotinylated strand is then resynthesized using a different adaptor-specific primer in the presence of a radiolabelled dNTP. The labelled immobilized 3´cDNA ends are next sequentially treated with a series of different restriction endonucleases and the products from each digestion analysed by PAGE. The result is a fingerprint composed of a number of ladders (equal to the number of sequential digests used). By comparing test versus control fingerprints, it is possible to identify differentially expressed products which can then be isolated from the gel and cloned. The advantages of this procedure are that it is very robust and reproducible, and the authors estimate that 80–93% of cDNA molecules are involved in the final fingerprint. The disadvantage is that polyacrylamide gels can rarely resolve more than 300–400 bands, which compares poorly to the 1000 or more which are estimated to be produced in an average experiment. The use of 2-D gels such as those described by Uitterlinden et al. (1989) and Hatada et al. (1991) may help to overcome this problem.

A similar method for displaying restriction endonuclease fragments was later described by Prashar and Weissman (1996). However, instead of sequential digestion of the immobolized 3'-terminal cDNA fragments, these authors simply compared the profiles of the control and treated populations without further manipulation.

AAAA
TTTT●

1st strand cDNA synthesis using
biotinylated poly dT primers

AAAA
TTTT●

↓ cDNA cleaved with AE and
captured with streptavivin beads

GTAC ——————AAAA
——————TTTT●

GTAC ——————AAAA
——————TTTT●

Divide in half and ligate linkers

CATG——————AAAA
GTAC——————TTTTT●

CATG——————AAAA
GTAC——————TTTTT●

CATG——————AAAA
GTAC——————TTTT●

CATG——————AAAA
GTAC——————TTTT●

Cleave with tagging enzyme (TE)
and produce blunt ends

GGATGCATGXXXXXXXXX
CCTACGTACXXXXXXXXX

TE   AE   Tag

GGATGCATGOOOOOOOOO
CCTACGTACOOOOOOOOO

TE   AE   Tag

↓ Ligate and amplify

GGATGCATGXXXXXXXXXOOOOOOOOOOCATGCATCC
CCTACGTACXXXXXXXXXOOOOOOOOOOGTACGTAGG

DiTag

Cleave with AE, isolate diTags,
concatenate, clone and
sequence

AE   AE   AE
---CATGXXXXXXXXXOOOOOOOOOOCATG XXXXXXXXXOOOOOOOOOOCATG---
---GTACXXXXXXXXXOOOOOOOOOOGTAC XXXXXXXXXOOOOOOOOOOGTAC---

Tag 1   Tag 2   Tag 3   Tag 4

Figure 9. Serial analysis of gene expression (SAGE) analysis. cDNA is cleaved with an anchoring enzyme (AE) and the 3′ends captured using streptavidin beads. The cDNA pool is divided in half and each portion ligated to a different linker, each containing a type IIS restriction site (tagging enzyme, TE). Restriction with the type IIS enzyme releases the linker plus a short length of cDNA (XXXXX and OOOOO indicate nucleotides of different tags). The two pools of tags are then ligated and amplified using linker-specific primers. Following PCR, the products are cleaved with the AE and the ditags isolated from the linkers using PAGE. The ditags are then ligated (during which process, concatenization occurs) and cloned into a vector of choice for sequencing. After Velculescu *et al.* (1995), with permission.

DNA arrays

'Open' differential display systems are cumbersome in that it takes a great deal of time to extract and identify candidate genes and then confirm that they are indeed up- or down-regulated in the treated compared to the control tissue. Normally, the latter process is carried out using Northern blotting or RT-PCR. Even so, each of the aforementioned steps produce a bottleneck to the ultimate goal of rapid analysis of gene expression. These problems will likely be addressed by the development of so-called DNA arrays (e.g. Gress *et al.* 1992, Zhao *et al.* 1995, Schena *et al.* 1996), the introduction of which has signalled the next era in differential gene expression analysis. DNA arrays consist of a gridded membrane or glass 'chips' containing hundreds or thousands of DNA spots, each consisting of multiple copies of part of a known gene. The genes are often selected based on previously proven involvement in oncogenesis, cell cycling, DNA repair, development and other cellular processes. They are usually chosen to be as specific as possible for each gene and animal species. Human and mouse arrays are already commercially available and a few companies will construct a personalized array to order, for example Clontech Laboratories and Research Genetics Inc. The technique is rapid in that hundreds or even thousands of genes can be spotted on a single array, and that mRNA/cDNA from the test populations can be labelled and used directly as probe. When analysed with appropriate hardware and software, arrays offer a rapid and quantitative means to assess differences in gene expression between two cell populations. Of course, there can only be identification and quantitation of those genes which are in the array (hence the term 'closed' system). Therefore, one approach to elucidating the molecular mechanisms involved in a particular disease/development system may be to combine an open and closed system—a DNA array to directly identify and quantitate the expression of known genes in mRNA populations, and an open system such as SSH to isolate unknown genes which are differentially expressed.

One of the main advantages of DNA arrays is the huge number of gene fragments which can be put on a membrane—some companies have reported gridding up to 60 000 spots on a single glass 'chip' (microscope slide). These high density chip-based micro-arrays will probably become available as mass-produced off-the-shelf items in the near future. This should facilitate the more rapid determination of differential expression in time and dose-response experiments. Aside from their high cost and the technical complexities involved in producing and probing DNA arrays, the main problem which remains, especially with the newer micro-array (gene-chip) technologies, is that results are often not wholly reproducible between arrays. However, this problem is being addressed and should be resolved within the next few years.

EST databases as a means to identify differentially expressed genes

Expressed sequence tags (ESTs) are partial sequences of clones obtained from cDNA libraries. Even though most ESTs have no formal identity (putative identification is the best to be hoped for), they have proven to be a rapid and efficient means of discovering new genes and can be used to generate profiles of gene-expression in specific cells. Since they were first described by Adams *et al.* (1991), there has been a huge explosion in EST production and it is estimated that there are now well over a million such sequences in the public domain, representing over half

of all human genes (Hillier *et al.* 1996). This large number of freely available sequences (both sequence information and clones are normally available royalty-free from the originators) has enabled the development of a new approach towards differential gene expression analysis as described by Vasmatzis *et al.* (1998). The approach is simple in theory: EST databases are first searched for genes that have a number of related EST sequences from the target tissue of choice, but none or few from non-target tissue libraries. Programmes to assist in the assembly of such sets of overlapping data may be developed in-house or obtained privately or from the internet. For example, the Institute for Genomic Research (TIGR, found at http://www.tigr.org) provides many software tools free of charge to the scientific community. Included amongst these is the TIGR assembler (Sutton *et al.* 1995), a tool for the assembly of large sets of overlapping data such as ESTs, bacterial artificial chromosomes (BAC)s, or small genomes. Candidate EST clones representing different genes are then analysed using RNA blot methods for size and tissue specificity and, if required, used as probes to isolate and identify the full length cDNA clone for further characterization. In practice however, the method is rather more involved, requiring bioinformatic and computer analysis coupled with confirmatory molecular studies. Vasmatzis *et al.* (1998) have described several problems in this fledgling approach, such as separating highly homologous sequences derived from different genes and an overemphasis of specificity for some EST sequences. However, since these problems will largely be addressed by the development of more suitable computer algorithms and an increased completeness of the EST database, it is likely that this approach to identifying differentially expressed genes may enjoy more patronage in the future.

Problems and potential of differential expression techniques

The holistic or single cell approach?

When working with *in vivo* models of differential expression, one of the first issues to consider must be the presence of multiple cell types in any given specimen. For example, a liver sample is likely to contain not only hepatocytes, but also (potentially) Ito cells, bile ductule cells, endothelial cells, various immune cells (e.g. lymphocytes, macrophages and Kupffer cells) and fibroblasts. Other tissues will each have their own distinctive cell populations. Also, in the case of neoplastic tissue, there are almost always normal, hyperplastic and/or dysplastic cells present in a sample. One must, therefore, be aware that genes obtained from a differential display experiment performed on an animal tissue model may not necessarily arise exclusively from the intended 'target' cells, e.g. hepatocytes/neoplastic cells. If appropriate, further analyses using immunohistochemistry, *in situ* hybridization or *in situ* RT-PCR should be used to confirm which cell types are expressing the gene(s) of interest. This problem is probably most acute for those studying the differential expression of genes in the development of different cell types, where there is a need to examine homologous cell populations. The problem is now being addressed at the National Cancer Institute (Bethesda, MD, USA) where new micro-disection techniques have been employed to assist in their gene analysis programme, the Cancer Genome Anatomy Project (CGAP) (For more information see web site: http://www.ncbi.nlm.nih.gov/ncicgap/intro.html). There are also separation techniques available that utilise cell-specific antigens as a means to isolate target cells,

e.g. fluorescence activated cell sorting (FACS) (Dunbar *et al.* 1998, Kas-Deelen *et al.* 1998) and magnetic bead technology (Richard *et al.* 1998, Rogler *et al.* 1998).

However, those taking a holistic approach may consider this issue unimportant. There is an equally appropriate view that all those genes showing altered expression within a compromized tissue should be taken into consideration. After all, since all tissues are complex mixes of different, interacting cell types which intimately regulate each other's growth and development, it is clear that each cell type could in some way contribute (positively or negatively) towards the molecular mechanisms which lie behind responses to external stimuli or neoplastic growth. It is perhaps then more informative to carry out differential display experiments using *in vivo* as opposed to *in vitro* models, where uniform populations of identical cells probably represent a partial, skewed or even inaccurate picture of the molecular changes that occur.

The incidence and possible implications of inter-individual biological variation should be considered in any approach where whole animal models are being used. It is clear that individuals (humans and animals) respond in different ways to identical stimuli. One of the best characterized examples is the debrisoquine oxidation polymorphism, which is mediated by cytochrome CYP2D6 and determines the pharmacokinetics of many commonly prescribed drugs (Lennard 1993, Meyer and Zanger 1997). The reasons for such differences are varied and complex, but allelic variations, regulatory region polymorphisms and even physical and mental health can all contribute to observed differences in individual responses. Careful thought should, therefore, be given to the specific objectives of the study and to the possible value of pooling starting material (tissue/mRNA). The effect of this can be beneficial through the ironing out of exaggerated responses and unimportant minor fluctuations of (mechanistically) irrelevant genes in individual animals, thus providing a clearer overall picture of the general molecular mechanisms of the response. However, at the same time such minor variations may be of utmost importance in deciding the ability of individual animals to succumb to or resist the effects of a given chemical/disease.

How efficient are differential expression techniques at recovering a high percentage of differentially expressed genes?

A number of groups have produced experimental data suggesting that mammalian cells produce between 8000–15 000 different mRNA species at any one time (Mechler and Rabbitts 1981, Hedrick *et al.* 1984, Bravo 1990), although figures as high as 20–30 000 have also been quoted (Axel *et al.* 1976). Hedrick *et al.* (1984) provided evidence suggesting that the majority of these belong to the rare abundance class. A breakdown of this abundance distribution is shown in table 1.

When the results of differential display experiments have been compared with data obtained previously using other methods, it is apparent that not all differentially expressed mRNAs are represented in the final display. In particular, rare messages (which, importantly, often include regulatory proteins) are not easily recovered using differential display systems. This is a major shortcoming, as the majority of mRNA species exist at levels of less than 0.005% of the total population (table 1). Bertioli *et al.* (1995) examined the efficiency of DD templates (heterogeneous mRNA populations) for recovering rare messages and were unable to detect mRNA

species present at less than 1.2% of the total mRNA population—equivalent to an intermediate or abundant species. Interestingly, when simple model systems (single target only) were used instead of a heterogeneous mRNA population, the same primers could detect levels of target mRNA down to 10000× smaller. These results are probably best explained by competition for substrates from the many PCR products produced in a DD reaction.

The numbers of differentially expressed mRNAs reported in the literature using various model systems provides further evidence that many differentially expressed mRNAs are not recovered. For example, DeRisi *et al.* (1997) used DNA array technology to examine gene expression in yeast following exhaustion of sugar in the medium, and found that more than 1700 genes showed a change in expression of at least 2-fold. In light of such a finding, it would not be unreasonable to suggest that of the 8000–15 000 different mRNA species produced by any given mammalian cell, up to 1000 or more may show altered expression following chemical stimulation. Whilst this may be an extreme figure, it is known that at least 100 genes are activated/upregulated in Jurkat (T-) cells following IL-2 stimulation (Ullman *et al.* 1990). In addition, Wan *et al.* (1996) estimated that interferon-γ-stimulated HeLa cells differentially express up to 433 genes (assuming 24000 distinct mRNAs expressed by the cells). However, there have been few publications documenting anywhere near the recovery of these numbers. For example, in using DD to compare normal and regenerating mouse liver, Bauer *et al.* (1993) found only 70 of 38000 total bands to be different. Of these, 50% (35 genes) were shown to correspond to differentially expressed bands. Chen *et al.* (1996) reported 10 genes upregulated in female rat liver following ethinyl estradiol treatment. McKenzie and Drake (1997) identified 14 different gene products whose expression was altered by phorbol myristate acetate (PMA, a tumour promoter agent) stimulation of a human myelomonocytic cell line. Kilty and Vickers (1997) identified 10 different gene products whose expression was upregulated in the peripheral blood leukocytes of allergic disease sufferers. Linskens *et al.* (1995) found 23 genes differentially expressed between young and senescent fibroblasts. Techniques other than DD have also provided an apparent paucity of differentially expressed genes. Using SH for example, Cao *et al.* (1997) found 15 genes differentially expressed in colorectal cancer compared to normal mucosal epithelium. Fitzpatrick *et al.* (1995) isolated 17 genes upregulated in rat liver following treatment with the peroxisome proliferator, clofibrate; Philips *et al.* (1990) isolated 12 cDNA clones which were upregulated in highly metastatic mammary adenocarcinoma cell lines compared to poorly metastatic ones. Prashar and Weissman (1996) used 3′ restriction fragment analysis and identified approximately 40 genes showing altered expression within 4 h of activation of Jurkat T-cells. Groenink and Leegwater (1996) analysed 27 gene fragments isolated using SSH of delayed early response phase of liver regeneration and found only 12 to be upregulated.

In the laboratory, SSH was used to isolate up to 70 candidate genes which appear to show altered expression in guinea pig liver following short-term treatment with the peroxisome proliferator, WY-14,643 (Rockett, Swales, Esdaile and Gibson, unpublished observations). However, these findings have still to be confirmed by analysis of the extracted tissue mRNA for differential expression of these sequences.

Whilst the latest differential display technologies are purported to include design and experimental modifications to overcome this lack of efficiency (in both the total number of differentially expressed genes recovered and the percentage that are true

positives), it is still not clear if such adaptations are practically effective—proving efficiency by spiking with a known amount of limited numbers of artificial construct(s) is one thing, but isolating a high percentage of the rare messages already present in an mRNA population is another. Of course, some models will genuinely produce only a small number of differentially expressed genes. In addition, there are also technical problems that can reduce efficiency. For example, mRNAs may have an unusual primary structure that effectively prevents their amplification by PCR-based systems. In addition, it is known that under certain circumstances not all mRNAs have 3′ polyA sites. For example, during *Xenopus* development, deadenylation is used as a means to stabilize RNAs (Voeltz and Steitz 1998), whilst preferential deadenylation may play a role in regulating Hsp70 (and perhaps, therefore, other stress protein) expression in *Drosophila* (Dellavalle *et al.* 1994). The presence of deadenylated mRNAs would clearly reduce the efficiency of systems utilizing a polydT reverse transcription step. The efficiency of any system also depends on the quality of the starting material. All differential display techniques use mRNA as their target material. However, it is difficult to isolate mRNA that is completely free of ribosomal RNA. Even if polydT primers are used to prime first strand cDNA synthesis, ribosomal RNA is often transcribed to some degree (Clontech PCR-Select cDNA Subtraction kit user manual). It has been shown, at least in the case of SSH, that a high rRNA:mRNA ratio can lead to inefficient subtractive hybridization (Clontech PCR-Select cDNA Subtraction kit user manual), and there is no reason to suppose that it will not do likewise in other SH approaches. Finally, those techniques that utilise a presubtraction amplification step (e.g. RDA) may present a skewed representation since some sequences amplify better than others.

Of course, probably the most important consideration is the temporal factor. It is clear that any given differential display experiment can only interrogate a cell at one point in time. It may well be that a high percentage of the genes showing altered expression at that time are obtained. However, given that disease processes and responses to environmental stimuli involve dynamic cascades of signalling, regulation, production and action, it is clear that all those genes which are switched on/off at different times will not be recovered and, therefore, vital information may well be missed. It is, therefore, imperative to obtain as much information about the model system beforehand as possible, from which a strategy can be derived for targeting specific time points or events that are of particular interest to the investigator. One way of getting round this problem of single time point analysis is to conduct the experiment over a suitable time course which, of course, adds substantially to the amount of work involved.

How sensitive are differential expression technologies?

There has been little published data that addresses the issue of how large the change in expression must be for it to permit isolation of the gene in question with the various differential expression technologies. Although the isolation of genes whose expression is changed as little as 1.5-fold has been reported using SSH (Groenink and Leegwater 1996), it appears that those demonstrating a change in excess of 5-fold are more likely to be picked up. Thus, there is a 'grey zone' in between where small changes could fade in and out of isolation between

experiments and animals. DD, on the other hand, is not subject to this grey zone since, unlike SH approaches, it does not amplify the difference in expression between two samples. Wan *et al.* (1996) reported that differences in expression of twofold or more are detectable using DD.

Resolution and visualization of differential expression products

It seems highly improbable with current technology that a gel system could be developed that is able to resolve all gene species showing altered expression in any given test system (be it SH- or DD-based). Polyacrylamide gel electrophoresis (PAGE) can resolve size differences down to 0.2% (Sambrook *et al.* 1989) and are used as standard in DD experiments. Even so, it is clear that a complex series of gene products such as those seen in a DD will contain unresolvable components. Thus, what appears to be one band in a gel may in fact turn out to be several. Indeed, it has been well documented (Mathieu-Daude *et al.* 1996, Smith *et al.* 1997) that a single band extracted from a DD often represents a composite of heterogeneous products, and the same has been found for SSH displays in this laboratory (Rockett *et al.* 1997). One possible solution was offered by Mathieu-Daude *et al.* (1996), who extracted and reamplified candidate bands from a DD display and used single strand conformation polymorphism (SSCP) analysis to confirm which components represented the truly differentially expressed product.

Many scientists often try to avoid the use of PAGE where possible because it is technically more demanding than agarose gel electrophoresis (AGE). Unfortunately, high resolution agarose gels such as Metaphor (FMC, Lichfield, UK) and AquaPor HR (National Diagnostics, Hessle, UK), whilst easier to prepare and manipulate than PAGE, can only separate DNA sequences which differ in size by around 1.5–2% (15–20 base pairs for a 1Kb fragment). Thus, SSH, RDA or other such products which differ in size by less than this amount are normally not resolvable. However, a simple technique does in fact exist for increasing the resolving power of AGE—the inclusion of HA-red (10-phenyl neutral red-PEG ligand) or HA-yellow (bisbenzamide-PEG ligand) (Hanse Analytik GmbH, Bremen, Germany) in a gel separates identical or closely sized products on base content. Specifically, HA-red and -yellow selectively bind to GC and AT DNA motifs, respectively (Wawer *et al.* 1995, Hanse Analytik 1997, personal communication). Since both HA-stains possess an overall positive charge, they migrate towards the cathode when an electric field is applied. This is in direct opposition to DNA, which is negatively charged and, therefore, migrates towards the anode. Thus, if two DNA clones are identical in size (as perceived on a standard high resolution agarose gel), but differ in AT/GC content, inclusion of a HA-dye in the gel will effectively retard the migration of one of the sequences compared to the other, effectively making it apparently larger and, thus, providing a means of differentiating between the two. The use of HA-red has been shown to resolve sequences with an AT variation of less than 1% (Wawer *et al.* 1995), whilst Hanse Analytik have reported that HA staining is so sensitive that in one case it was used to distinguish two 567bp sequences which differed by only a single point mutation (Hanse Analytik 1996, personal communication). Therefore, if one wishes to check whether all the clones produced from a specific band in a differential display experiment are derived from the same gene species, a small amount of reamplified or digested clone can be run on a standard high resolution gel, and a second aliquot
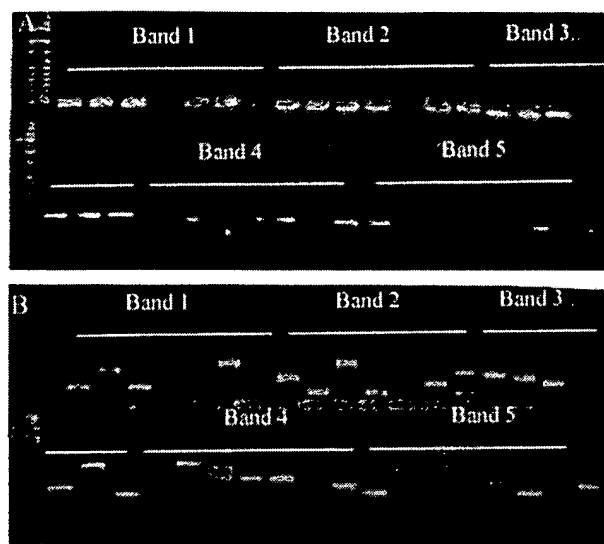
Figure 10. Discrimination of clones of identical/nearly identical size using HA-red. Bands of decreasing size (1–5) were extracted from the final display of a suppression subtractive hybridization experiment and cloned. Seven colonies were picked at random from each cloned band and their inserts amplified using PCR. The products were run on two gels, (A) a high resolution 2% agarose gel, and (B) a high resolution 2% agarose gel containing 1 U/ml HA-red. With few exceptions, all the clones from each band appear to be the same size (gel A). However, the presence of HA-red (gel B), which separates identically-sized DNA fragments based on the percentage of GC within the sequence, clearly indicates the presence of different gene species within each band. For example, even though all five re-amplified clones of band 1 appear to be the same size, at least four different gene species are represented.

in a similar gel containing one of the HA-stains. The standard gel should indicate any gross size differences, whilst the HA-stained gel should separate otherwise unresolvable species (on standard AGE) according to their base content. Geisinger *et al.* (1997) reported successful use of this approach for identifying DD-derived clones. Figure 10 shows such an experiment carried out in this laboratory on clones obtained from a band extracted from an SSH display.

An alternative approach is to carry out a 2-D analysis of the differential display products. In this approach, size-based separation is first carried out in a standard agarose gel. The gel slice containing the display is then extracted and incorporated in to a HA gel for resolution based on AT/GC content.

Of course, one should always consider the possibility of there being different gene species which are the same size and have the same GC/AT content. However, even these species are not unresolvable given some effort—again, one might use SSCP, or perhaps a denaturing gradient gel electrophoresis (DGGE) or temperature gradient field electrophoresis (TGGE) approach to resolve the contents of a band, either directly on the extracted band (Suzuki *et al.* 1991) or on the reamplified product.

The requirement of some differential display techniques to visualize large numbers of products (e.g. DD and GEF) can also present a problem in that, in terms of numbers, the resolution of PAGE rarely exceeds 300–400 bands. One approach to overcoming this might be to use 2-D gels such as those described by Uitterlinden *et al.* (1989) and Hatada *et al.* (1991).

Extraction of differentially expressed bands from a gel can be complex since, in some cases (e.g. DD, GEF), the results are visualized by autoradiographic means, such that precise overlay of the developed film on the gel must occur if the correct band is to be extracted for further analysis. Clearly, a misjudged extraction can account for many man-hours lost. This problem, and that of the use of radioisotopes, has been addressed by several groups. For example, Lohmann *et al.* (1995) demonstrated that silver staining can be used directly to visualize DD bands in horizontal PAGs. An *et al.* (1996) avoided the use of radioisotopes by transferring a small amount (20–30%) of the DNA from their DD to a nylon membrane, and visualizing the bands using chemiluminescent staining before going back to extract the remaining DNA from the gel. Chen and Peck (1996) went one step further and transferred the entire DD to a nylon membrane. The DNA bands were then visualized using a digoxigenin (DIG) system (DIG was attached to the polydT primers used in the differential display procedure). Differentially expressed bands were cut from the membrane and the DNA eluted by washing with PCR buffer prior to reamplification.

One of the advantages of using techniques such as SSH and RDA is that the final display can be run on an agarose gel and the bands visualized with simple ethidium bromide staining. Whilst this approach can provide acceptable results, overstaining with SYBR Green I or SYBR Gold nucleic acid stains (FMC) effectively enhances the intensity and sharpness of the bands. This greatly aids in their precise extraction and often reveals some faint products that may otherwise be overlooked. Whilst differential displays stained with SYBR Green I are better visualized using short wavelength UV (254 nm) rather than medium wavelength (306 nm), the shorter wavelength is much more DNA damaging. In practice, it takes only a few seconds to damage DNA extracted under 254 nm irradiation, effectively preventing reamplification and cloning. The best approach is to overstain with SYBR Green I and extract bands under a medium wavelength UV transillumination.

The possible use of 'microfingerprinting' to reduce complexity

Given the sheer number of gene products and the possible complexity of each band, an alternative approach to rapid characterization may be to use an enhanced analysis of a small section of a differential display—a 'sub-fingerprint' or 'micro-fingerprint'. In this case, one could concentrate on those bands which only appear in a particular chosen size region. Reducing the fingerprint in this way has at least two advantages. One is that it should be possible to use different gel types, concentrations and run times tailored exactly to that region. Currently, one might run products from 100–3000 + bp on the same gel, which leads to compromize in the gel system being used and consequently to suboptimal resolution, both in terms of size and numbers, and can lead to problems in the accurate excision of individual bands. Secondly, it may be possible to enhance resolution by using a 2-D analysis using a HA-stain, as described earlier. In summary, if a range of gene product sizes is carefully chosen to included certain 'relevant' genes, the 2-D system standardized, and appropriate gene analysis used, it may be possible to develop a method for the early and rapid identification of compounds which have similar or widely different cellular effects. If the prognosis for exposure to one or more other chemicals which display a similar profile is already known, then one could perhaps predict similar effects for any new compounds which show a similar micro-fingerprint.

An alternative approach to microfingerprinting is to examine altered expression in specific families of genes through careful selection of PCR primers and/or post-reaction analysis. Stress genes, growth factors and/or their receptors, cell cycling genes, cytochromes P450 and regulatory proteins might be considered as candidates for analysis in this way. Indeed, some off-the-shelf DNA arrays (e.g. Clontech's Atlas cDNA Expression Array series) already anticipated this to some degree by grouping together genes involved in different responses e.g. apoptosis, stress, DNA-damage response etc.

Screening

False positives

The generation of false positives has been discussed at length amongst the differential display community (Liang *et al.* 1993, 1995, Nishio *et al.* 1994, Sun *et al.* 1994, Sompayrac *et al.* 1995). The reason for false positives varies with the technique being used. For instance, in RDA, the use of adaptors which have not been HPLC purified can lead to the production of false positives through illegitimate ligation events (O'Neill and Sinclair 1997), whilst in DD they can arise through PCR artifacts and illegitemate transcription of rRNA. In SH, false positives appear to be derived largely from abundant gene species, although some may arise from cDNA/mRNA species which do not undergo hybridization for technical reasons.

A quick screening of putative differentially expressed clones can be carried out using a simple dot blot approach, in which labelled first strand probes synthesized from tester and driver mRNA are hybridized to an array of said clones (Hedrick *et al.* 1984, Sakaguchi *et al.* 1986). Differentially expressed clones will hybridize to tester probe, but not driver. The disadvantage of this approach is that rare species may not generate detectable hybridization signals. One option for those using SSH is to screen the clones using a labelled probe generated from the subtracted cDNA from which it was derived, and with a probe made from the reverse subtraction reaction (ClonTechniques 1997a). Since the SSH method enriches rare sequences, it should be possible to confirm the presence of clones representing low abundance genes. Despite this quick screening step, there is still the need to go back to the original mRNA and confirm the altered expression using a more quantitative approach. Although this may be achieved using Northern blots, the sensitivity is poor by today's high standards and one must rely on PCR methods for accurate and sensitive determinations (see below).

Sequence analysis

The majority of differential display procedures produce final products which are between 100 and 1000bp in size. However, this may considerably reduce the size of the sequence for analysis of the DNA databases. This in turn leads to a reduced confidence in the result—several families of genes have members whose DNA sequences are almost identical except in a few key stretches, e.g. the cytochrome P450 gene superfamily (Nelson *et al.* 1996). Thus, does the clone identified as being almost identical to gene X_0 really come from that gene, or its brother gene X_1 or its as yet undiscovered sister X_2? For example, using SSH, part of a gene was isolated,

which was up-regulated in the liver of rats exposed to Wy-14,643 and was identified by a FASTA search as being transferrin (data not shown). However, transferrin is known to be downregulated by hypolipidemic peroxisome proliferators such as Wy-14,643 (Hertz *et al.* 1996), and this was confirmed with subsequent RT-PCR analysis. This suggests that the gene sequence isolated may belong to a gene which is closely related to transferrin, but is regulated by a different mechanism.

A further problem associated with SH technology is redundancy. In most cases before SH is carried out, the cDNA population must first be simplified by restriction digestion. This is important for at least two reasons:

(1) To reduce complexity—long cDNA fragments may form complex networks which prevent the formation of appropriate hybrids, especially at the high concentrations required for efficient hybridization.

(2) Cutting the cDNAs into small fragments provides better representation of individual genes. This is because genes derived from related but distinct members of gene families often have similar coding sequences that may cross-hybridize and be eliminated during the subtraction procedure (Ko 1990). Furthermore, different fragments from the same cDNA may differ considerably in terms of hybridization and amplification and, thus, may not efficiently do one or the other (Wang and Brown 1991). Thus, some fragments from differentially expressed cDNAs may be eliminated during subtractive hybridization procedures. However, other fragments may be enriched and isolated. As a consequence of this, some genes will be cut one or more times, giving rise to two or more fragments of different sizes. If those same genes are differentially expressed, then two or more of the different size fragments may come through as separate bands on the final differential display, increasing the observed redundancy and increasing the number of redundant sequencing reactions.

Sequence comparisons also throw up another important point—at what degree of sequence similarity does one accept a result. Is 90% identitiy between a gene derived from your model species and another acceptably close? Is 95% between your sequence and one from the same species also acceptable? This problem is particularly relevant when the forward and reverse sequence comparisons give similar sequences with completely different gene species! An arbitrary decision seems to be to allocate genes that are definite (95% and above similarity) and then group those between 60 and 95% as being related or possible homologues.

Quantitative analysis

At some point, one must give consideration to the quantitative analysis of the candidate genes, either as a means of confirming that they are truly differentially expressed, or in order to establish just what the differences are. Northern blot analysis is a popular approach as it is relatively easy and quick to perform. However, the major drawback with Northern blots is that they are often not sensitive enough to detect rare sequences. Since the majority of messages expressed in a cell are of low abundance (see table 1), this is a major problem. Consequently, RT-PCR may be the method of choice for confirming differential expression. Although the procedure is somewhat more complex than Northern analysis, requiring synthesis of primers and optimization of reaction conditions for each gene species, it is now possible to set up high throughput PCR systems using mulitchannel pipettes, 96 +-well plates and

appropriate thermal cycling technology. Whilst quantitative analysis is more desirable, being more accurate and without reliance on an internal standard, the money and time needed to develop a competitor molecule is often excessive, especially when one might be examining tens or even hundreds of gene species. The use of semi-quantitative analysis is simpler, although still relatively involved. One must first of all choose an internal standard that does not change in the test cells compared to the controls. Numerous reference genes have been tried in the past, for example interferon-gamma (IFN-γ, Frye *et al.* 1989), β-actin (Heuval *et al.* 1994), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Wong *et al.* 1994), dihydrofolate reductase (DHFR, Mohler and Butler 1991), β-2-microglobulin (β-2-m, Murphy *et al.* 1990), hypoxanthine phosphoribosyl transferase (HPRT, Foss *et al.* 1998) and a number of others (ClonTechniques 1997b). Ideally, an internal standard should not change its level of expression in the cell regardless of cell age, stage in the cell cycle or through the effects of external stimuli. However, it has been shown on numerous occasions that the levels of most housekeeping genes currently used by the research community do in fact change under certain conditions and in different tissues (ClonTechniques 1997b). It is imperative, therefore, that preliminary experiments be carried out on a panel of housekeeping genes to establish their suitability for use in the model system.

Interpretation of quantitative data must also be treated with caution. By comparing the lists of genes identified by differential expression one can perhaps gain insight into why two different species react in different ways to external stimuli. For example, rats and mice appear sensitive to the non-genotoxic effects of a wide range of peroxisome proliferators whilst Syrian hamsters and guinea pigs are largely resistant (Orton *et al.* 1984, Rodricks and Turnbull 1987, Lake *et al.* 1989, 1993, Makowska *et al.* 1992). A simplified approach to resolving the reason(s) why is to compare lists of up- and down-regulated genes in order to identify those which are expressed in only one species and, through background knowledge of the effects of the said gene, might suggest a mechanism of facilitated non-genotoxic carcinogenesis or protection. Of course, the situation is likely to be far more complex. Perhaps if there were one key gene protecting guinea pig from non-genotoxic effects and it was upregulated 50 times by PPs, the same gene might only be up-regulated five times in the rat. However, since both were noted to be upregulated, the importance of the gene may be overlooked. Just to complicate matters, a large change in expression does not necessarily mean a biologically important change. For example, what is the true relevance of gene Y which shows a 50-fold increase after a particular treatment, and gene Z which shows only a 5-fold increase? If one examines the literature one may find that historically, gene Y has often been shown to be up-regulated 40–60-fold by a number of unrelated stimuli—in light of this the 50-fold increase would appear less significant. However, the literature may show that gene Z has never been recorded as having more than doubled in expression—which makes your 5-fold increase all the more exciting. Perhaps even more interesting is if that same 5-fold increase has only been seen in related neoplasms or following treatment with related chemicals.

Problems in using the differential display approach

Differential display technology originally held promise of an easily obtainable 'fingerprint' of those genes which are up- or down-regulated in test animals/cells in a developmental process or following exposure to given stimuli. However, it has

become clear that the fingerprinting process, whilst still valid, is much too complex to be represented by a single technique profile. This is because all differential display techniques have common and/or unique technical problems which preclude the isolation and identification of all those genes which show changes in expression. Furthermore, there are important genetic changes related to disease development which differential expression analysis is simply not designed to address. An example of this is the presence of small deletions, insertions, or point mutations such as those seen in activated oncogenes, tumour suppressor genes and individual poly-morphisms. Polymorphic variations, small though they usually are, are often regarded as being of paramount importance in explaining why some patients respond better than others to certain drug treatments (and, in logical extension, why some people are less affected by potentially dangerous xenobiotics/carcinogens than others). The identification of such point mutations and naturally occurring polymorphisms requires the subsequent application of sequencing, SSCP, DGGE or TGGE to the gene of interest. Furthermore, differential display is not designed to address issues such as alternatively spliced gene species or whether an increased abundance of mRNA is a result of increased transcription or increased mRNA stability.

Conclusions

Perhaps the main advantage of open system differential display techniques is that they are not limited by extant theories or researcher bias in revealing genes which are differentially expressed, since they are designed to amplify all genes which demonstrate altered expression. This means that they are useful for the isolation of previously unknown genes which may turn out be useful biomarkers of a particular state or condition. At least one open system (SAGE) is also quantitative, thus eliminating the need to return to the original mRNA and carry out Northern/PCR analysis to confirm the result. However, the rapid progress of genome mapping projects means that over the next 5–10 years or so, the balance of experimental use will switch from open to closed differential display systems, particularly DNA arrays. Arrays are easier and faster to prepare and use, provide quantitative data, are suitable for high throughput analysis and can be tailored to look at specific signalling pathways or families of genes. Identification of all the gene sequences in human and common laboratory animals combined with improved DNA array technology, means that it will soon no longer be necessary to try to isolate differentially expressed genes using the technically more demanding open system approach. Thus, their main advantage (that of identifying unknown genes) will be largely eradicated. It is likely, therefore, that their sphere of application will be reduced to analysis of the less common laboratory species, since it will be some time yet before the genomes of such animals as zebrafish, electric eels, gerbils, crayfish and squid, for example, will be sequenced.

Of course, in the end the question will always remain: What is the functional/biological significance of the identified, differentially expressed genes? One persistent problem is understanding whether differentially expressed genes are a cause or consequence of the altered state. Furthermore, many chemicals, such as non-genotoxic carcinogens, are also mitogens and so genes associated with replication will also be upregulated but may have little or nothing to do with the

carcinogenic effect. Whilst differential display technology cannot hope to answer these questions, it does provide a springboard from which identification, regulatory and functional studies can be launched. Understanding the molecular mechanism of cellular responses is almost impossible without knowing the regulation and function of those genes and their condition (e.g. mutated). In an abstract sense, differential display can be likened to a still photograph, showing details of a fixed moment in time. Consider the Historian who knows the outcome of a battle and the placement and condition of the troops before the battle commenced, but is asked to try and deduce how the battle progressed and why it ended as it did from a few still photographs—an impossible task. In order to understand the battle, the Historian must find out the capabilities and motivation of the soldiers and their commanding officers, what the orders were and whether they were obeyed. He must examine the terrain, the remains of the battle and consider the effects the prevailing weather conditions exerted. Likewise, if mechanistic answers are to be forthcoming, the scientist must use differential display in combination with other techniques, such as knockout technology, the analysis of cell signalling pathways, mutation analysis and time and dose response analyses. Although this review has emphasized the importance of differential gene profiling, it should not be considered in isolation and the full impact of this approach will be strengthened if used in combination with functional genomics and proteomics (2-dimensional protein gels from isoelectric focusing and subsequent SDS electrophoresis and virtual 2D-maps using capillary electrophoresis). Proteomics is attracting much recent attention as many of the changes resulting in differential gene expression do not involve changes in mRNA levels, as decribed extensively herein, but rather protein–protein, protein–DNA and protein phosphorylation events which would require functional genomics or proteomic technologies for investigation.

Despite the limitations of differential display technology, it is clear that many potential applications and benefits can be obtained from characterizing the genetic changes that occur in a cell during normal and disease development and in response to chemical or biological insult. In light of functional data, such profiling will provide a 'fingerprint' of each stage of development or response, and in the long term should help in the elucidation of specific and sensitive biomarkers for different types of chemical/biological exposure and disease states. The potential medical and therapeutic benefits of understanding such molecular changes are almost im-measurable. Amongst other things, such fingerprints could indicate the family or even specific type of chemical an individual has been exposed to plus the length and/or acuteness of that exposure, thus indicating the most prudent treatment. They may also help uncover differences in histologically identical cancers, provide diagnostic tests for the earliest stages of neoplasia and, again, perhaps indicate the most efficacious treatment.

The Human Genome Project will be completed early in the next century and the DNA sequence of all the human genes will be known. The continuing development and evolution of differential gene expression technology will ensure that this knowledge contributes fully to the understanding of human disease processes.

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Whole genome analysis: Experimental access to all genome sequenced segments through larger-scale efficient oligonucleotide synthesis and PCR

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ABSTRACT   The recent ability to sequence whole genomes allows ready access to all genetic material. The approaches outlined here allow automated analysis of sequence for the synthesis of optimal primers in an automated multiplex oligonucleotide synthesizer (AMOS). The efficiency is such that all ORFs for an organism can be amplified by PCR. The resulting amplicons can be used directly in the construction of DNA arrays or can be cloned for a large variety of functional analyses. These tools allow a replacement of single-gene analysis with a highly efficient whole-genome analysis.

The genome sequencing projects have generated and will continue to generate enormous amounts of sequence data. The genomes of *Saccharomyces cerevisiae*, *Escherichia coli*, *Haemophilus influenzae* (1), *Mycoplasma genitalium* (2), and *Methanococcus jannaschii* (3) have been completely sequenced. Other model organisms have had substantial portions of their genomes sequenced as well, including the nematode *Caenorhabditis elegans* (4) and the small flowering plant *Arabidopsis thaliana* (5). This massive and increasing amount of sequence information allows the development of novel experimental approaches to identify gene function.

One standard use of genome sequence data is to attempt to identify the functions of predicted open reading frames (ORFs) within the genome by comparison to genes of known function. Such a comparative analysis of all ORFs to existing sequence data is fast, simple, and requires no experimentation and is therefore a reasonable first step. While finding sequence homologies/motifs is not a substitute for experimentation, noting the presence of sequence homology and/or sequence motifs can be a useful first step in finding interesting genes, in designing experiments and, in some cases, predicting function. However, this type of analysis is frequently uninformative. For example, over one-half of new ORFs in *S. cerevisiae* have no known function (6). If this is the case in a well studied organism such as yeast, the problem will be even worse in organisms that are less well studied or less manipulable. A large, experimentally determined gene function database would make homology/motif searches much more useful.

Experimental analysis must be performed to thoroughly understand the biological function of a gene product. Scaling up from classical "cottage industry" one-gene-oriented approaches to whole-genome analysis would be very expensive and laborious. It is clear that novel strategies are necessary to efficiently pursue the next phase of the genome projects—whole-genome experimental analysis to explore gene expression, gene product function, and other genome functions. Model organisms, such as *S. cerevisiae*, will be extremely important in the development of novel whole-genome analysis techniques and, subsequently, in improving our understanding of other more complex and less manipulable organisms.

The genome sequence can be systematically used as a tool to understand ORFs, gene product function, and other genome regions. Toward this end, a directed strategy has been developed for exploiting sequence information as a means of providing information about biological function (Fig. 1). Efforts have been directed toward the amplification of each predicted ORF or any other region of the genome ranging from a few base pairs to several kilobase pairs. There are many uses for these amplicons—they can be cloned into standard vectors or specialized expression vectors, or can be cloned into other specialized vectors such as those used for two-hybrid analysis. The amplicons can also be used directly by, for example, arraying onto glass for expression analysis, for DNA binding assays, or for any direct DNA assay (7). As a pilot study, synthetic primers were made on the 96-well automated multiplex oligonucleotide synthesizer (AMOS) instrument (8) (Fig. 2). These oligonucleotides were used to amplify each ORF on yeast chromosome V. The current version of this instrument can synthesize three plates of 96 oligonucleotides each (25 bases) in an 8-hr day. The amplification of the entire set of PCR products was then analyzed by gel electrophoresis (Fig. 3). Successful amplification of the proper length product on the first attempt was 95%. This project demonstrates that one can go directly from sequence information to biological analysis in a truly automated, totally directed manner.

These amplicons can be incorporated directly in arrays or the amplicons can be cloned. If the amplicons are to be cloned, novel sequences can be incorporated at the 5' end of the oligonucleotide to facilitate cloning. One potential problem with cloning PCR products is that the cloned amplicons may contain sequence alterations that diminish their utility. One option would be to resequence each individual amplicon. However, this is expensive, inefficient, and time consuming. A faster, more cost-effective, and more accurate approach is to apply comparative sequencing by denaturing HPLC (9). This method is capable of detecting a single base change in a 2-kb heteroduplex. Longer amplicons can be analyzed by use of appropriate restriction fragments. If any change is detected in a clone, an alternate clone of the same region can be analyzed. Modifying the system to allow high throughput analysis by denaturing HPLC is also relatively simple and straightforward.

If amplicons are used directly on arrays without cloning, it is important to note that, even if single PCR product bands are observed on gels, the PCR products will be contaminated with various amounts of other sequences. This contamination has the potential to affect the results in, for example, expression

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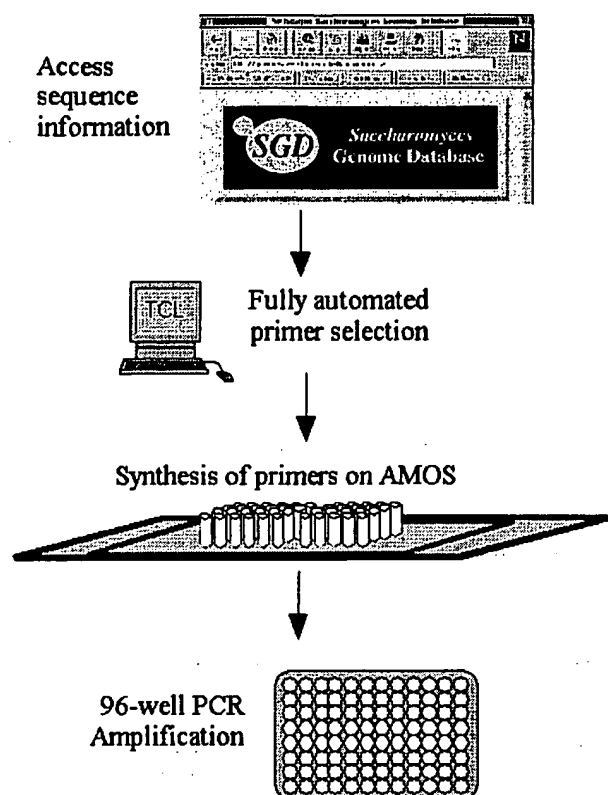
FIG. 1. Overview of systematic method for isolating individual genes. Sequence information is obtained automatically from sequence databases. The data are input into primer selection software specifically designed to target ORFs as designated by database annotations. The output file containing the primer information is directly read by a high-throughput oligonucleotide synthesizer, which makes the oligonucleotides in 96-well plates (AMOS, automated multiplex oligonucleotide synthesizer). The forward and reverse primers are synthesized in the same location on separate plates to facilitate the downstream handling of primers. The amplicons are generated by PCR in 96-well plates as well.

FIG. 2. Overall approach for using database of a genome to direct biological analysis. The synthesis of the 6,000 ORFs (orfs) for each gene of *S. cerevisiae* can be used in many applications utilizing both cloning and microarraying technology.

analysis. On the other hand, direct use of the amplicons is much less labor intensive and greatly decreases the occurrence of mistakes in clone identification, a ubiquitous problem associated with large clone set archiving and retrieving.

Any large-scale effort to capture each ORF within a genome must rely on automation if cost is to be minimized while efficiency is maximized. Toward that end, primers targeting ORFs were designed automatically using simple new scripts and existing primer selection software. These script-selected primer sequences were directly read by the high-throughput synthesizer and the forward and reverse primers were synthesized in separate plates in corresponding wells to facilitate automated pipetting and PCR amplifications. Each of the resulting PCR products, generated with minimum labor, contains a known, unique ORF.

Large-scale genome analysis projects are dependent on newly emerging technologies to make the studies practical and economically feasible. For example, the cost of the primers, a significant issue in the past, has been reduced dramatically to make feasible this and other projects that require tens of thousands of oligonucleotides. Other methods of high-throughput analysis are also vital to the success of functional analysis projects, such as microarraying and oligonucleotide chip methods (10–14).

Changes in attitude are also required. One of the major costs of commercial oligonucleotides is extensive quality control such that virtually 100% of the supplied oligonucleotides are successfully synthesized and work for their intended purpose.

Considerable cost reduction can be obtained by simply decreasing the expected successful synthesis rate to 95–97%. One can then achieve faster and cheaper whole genome coverage by simply adding a single quality control at the end of the experiment and batching the failures for resynthesis.

The directed nature of the amplicon approach is of clear advantage. The sequence of each ORF is analyzed automatically, and unique specific primers are made to target each ORF. Thus, there is relatively little time or labor involved—for example, no random cloning and subsequent screening is required because each product is known. In the test system, primers for 240 ORFs from chromosome V were systematically synthesized, beginning from the left arm and continuing through to the right arm. At no point was there any manual analysis of sequence information to generate the collection. In many ways, now that the sequence is known, there is no need for the researcher to examine it.

These amplicons can be arrayed and expression analysis can be done on all arrayed ORFs with a single hybridization (10). Those ORFs that display significant differential expression patterns under a given selection are easily identified without the laborious task of searching for and then sequencing a clone. Once scaled up, the procedure provides even greater returns on effort, because a single hybridization will ultimately provide a "snapshot" of the expression of all genes in the yeast genome. Thus, the limiting factor in whole genome analysis will not be the analysis process itself, but will instead be the ability of researchers to design and carry out experimental selections.

Current expression and genetic analysis technologies are geared toward the analysis of single genes and are ill suited to analyze numerous genes under many conditions. Additional difficulties with current technologies include: the effort and expense required to analyze expression and make mutants, the potential duplication of effort if done by different laboratories, and the possibility of conflicting results obtained from different laboratories. In contrast, whole genome analysis not only is more efficient, it also provides data of much higher quality; all genes are assayed and compared in parallel under exactly

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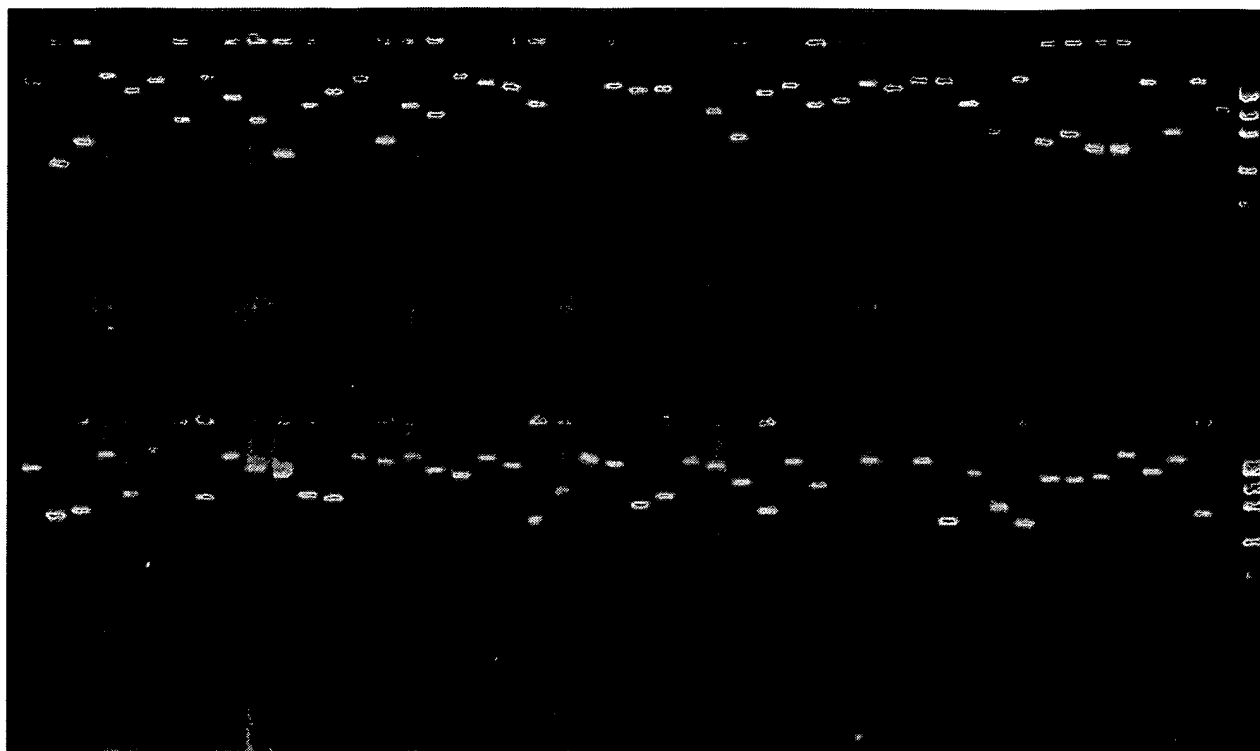
FIG. 3.   Gel image of amplifications. Using the method described in Fig. 1, amplicons were generated for ORFs of *S. cerevisiae* chromosome V. One plate of 96 amplification reactions is shown.

the same conditions. In addition, amplicons have many applications beyond gene expression. For example, one recent approach is to incorporate a unique DNA sequence tag, synthesized as part of each gene specific primer, during amplification. The tags or molecular bar codes, when reintroduced into the organism as a gene deletion or as a gene clone, can be used much more efficiently than individual mutations or clones because pools of tagged mutants or transformants can be analyzed in parallel. This parallel analysis is possible because the tags are readily and quantitatively amplified even in complex mixtures of tags (13).

These ORF genome arrays and oligonucleotide tagged libraries can be used for many applications. Any conventional selection applied to a library that gives discrete or multiple products can use these technologies for a simple direct readout. These include screens and selections for mutant complementation, overexpression suppression (15, 16), second-site suppressors, synthetic lethality, drug target overexpression (17), two-hybrid screens (18), genome mismatch scanning (19), or recombination mapping.

The genome projects have provided researchers with a vast amount of information. These data must be used efficiently and systematically to gain a truly comprehensive understanding of gene function and, more broadly, of the entire genome which can then be applied to other organisms. Such global approaches are essential if we are to gain an understanding of the living cell. This understanding should come from the viewpoint of the integration of complex regulatory networks, the individual roles and interactions of thousands of functional gene products, and the effect of environmental changes on both gene regulatory networks and the roles of all gene products. The time has come to switch from the analysis of a single gene to the analysis of the whole genome.

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■ IN PERSPECTIVE ■
Claudio J. Conti, Editor

Microarrays and Toxicology: The Advent of Toxicogenomics

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The availability of genome-scale DNA sequence information and reagents has radically altered life-science research. This revolution has led to the development of a new scientific subdiscipline derived from a combination of the fields of toxicology and genomics. This subdiscipline, termed toxicogenomics, is concerned with the identification of potential human and environmental toxicants, and their putative mechanisms of action, through the use of genomics resources. One such resource is DNA microarrays or "chips," which allow the monitoring of the expression levels of thousands of genes simultaneously. Here we propose a general method by which gene expression, as measured by cDNA microarrays, can be used as a highly sensitive and informative marker for toxicity. Our purpose is to acquaint the reader with the development and current state of microarray technology and to present our view of the usefulness of microarrays to the field of toxicology. *Mol. Carcinog. 24:153–159, 1999.* © 1999 Wiley-Liss, Inc.

Key words: toxicology; gene expression; animal bioassay

INTRODUCTION

Technological advancements combined with intensive DNA sequencing efforts have generated an enormous database of sequence information over the past decade. To date, more than 3 million sequences, totaling over 2.2 billion bases [1], are contained within the GenBank database, which includes the complete sequences of 19 different organisms [2]. The first complete sequence of a free-living organism, *Haemophilus influenzae,* was reported in 1995 [3] and was followed shortly thereafter by the first complete sequence of a eukaryote, *Saccharomyces cervisiae* [4]. The development of dramatically improved sequencing methodologies promises that complete elucidation of the *Homo sapiens* DNA sequence is not far behind [5].

To exploit more fully the wealth of new sequence information, it was necessary to develop novel methods for the high-throughput or parallel monitoring of gene expression. Established methods such as northern blotting, RNAse protection assays, S1 nuclease analysis, plaque hybridization, and slot blots do not provide sufficient throughput to effectively utilize the new genomics resources. Newer methods such as differential display [6], high-density filter hybridization [7,8], serial analysis of gene expression [9], and cDNA- and oligonucleotide-based microarray "chip" hybridization [10–12] are possible solutions to this bottleneck. It is our belief that the microarray approach, which allows the monitoring of expression levels of thousands of genes simultaneously, is a tool of unprecedented power for use in toxicology studies.

Almost without exception, gene expression is altered during toxicity, as either a direct or indirect result of toxicant exposure. The challenge facing toxicologists is to define, under a given set of experimental conditions, the characteristic and specific pattern of gene expression elicited by a given toxicant. Microarray technology offers an ideal platform for this type of analysis and could be the foundation for a fundamentally new approach to toxicology testing.

MICROARRAY DEVELOPMENT AND APPLICATIONS

cDNA Microarrays

In the past several years, numerous systems were developed for the construction of large-scale DNA arrays. All of these platforms are based on cDNAs or oligonucleotides immobilized to a solid support. In the cDNA approach, cDNA (or genomic) clones of interest are arrayed in a multi-well format and amplified by polymerase chain reaction. The products of this amplification, which are usually 500- to 2000-bp clones from the 3′ regions of the genes of interest, are then spotted onto solid support by using high-speed robotics. By using this method, microarrays of up to 10 000 clones can be generated by spotting onto a glass substrate

[13,14]. Sample detection for microarrays on glass involves the use of probes labeled with fluorescent or radioactive nucleotides.

Fluorescent cDNA probes are generated from control and test RNA samples in single-round reverse-transcription reactions in the presence of fluorescently tagged dUTP (e.g., Cy3-dUTP and Cy5-dUTP), which produces control and test products labeled with different fluors. The cDNAs generated from these two populations, collectively termed the "probe," are then mixed and hybridized to the array under a glass coverslip [10,11,15]. The fluorescent signal is detected by using a custom-designed scanning confocal microscope equipped with a motorized stage and lasers for fluor excitation [10,11,15]. The data are analyzed with custom digital image analysis software that determines for each DNA feature the ratio of fluor 1 to fluor 2, corrected for local background [16,17]. The strength of this approach lies in the ability to label RNAs from control and treated samples with different fluorescent nucleotides, allowing for the simultaneous hybridization and detection of both populations on one microarray. This method eliminates the need to control for hybridization between arrays. The research groups of Drs. Patrick Brown and Ron Davis at Stanford University spearheaded the effort to develop this approach, which has been successfully applied to studies of *Arabidopsis thaliana* RNA [10], yeast genomic DNA [15], tumorigenic versus non-tumorigenic human tumor cell lines [11], human T-cells [18], yeast RNA [19], and human inflammatory disease–related genes [20]. The most dramatic result of this effort was the first published account of gene expression of an entire genome, that of the yeast *Saccharomyces cervisiae* [21].

In an alternative approach, large numbers of cDNA clones can be spotted onto a membrane support, albeit at a lower density [7,22]. This method is useful for expression profiling and large-scale screening and mapping of genomic or cDNA clones [7,22–24]. In expression profiling on filter membranes, two different membranes are used simultaneously for control and test RNA hybridizations, or a single membrane is stripped and reprobed. The signal is detected by using radioactive nucleotides and visualized by phosphorimager analysis or autoradiography. Numerous companies now sell such cDNA membranes and software to analyze the image data [25–27].

Oligonucleotide Microarrays

Oligonucleotide microarrays are constructed either by spotting prefabricated oligos on a glass support [13] or by the more elegant method of direct in situ oligo synthesis on the glass surface by photolithography [28–30]. The strength of this approach lies in its ability to discriminate DNA molecules based on single base-pair difference. This allows the application of this method to the fields of medical diagnostics, pharmacogenetics, and sequencing by hybridization as well as gene-expression analysis.

Fabrication of oligonucleotide chips by photolithography is theoretically simple but technically complex [29,30]. The light from a high-intensity mercury lamp is directed through a photolithographic mask onto the silica surface, resulting in deprotection of the terminal nucleotides in the illuminated regions. The entire chip is then reacted with the desired free nucleotide, resulting in selected chain elongation. This process requires only 4n cycles (where n = oligonucleotide length in bases) to synthesize a vast number of unique oligos, the total number of which is limited only by the complexity of the photolithographic mask and the chip size [29,31,32].

Sample preparation involves the generation of double-stranded cDNA from cellular poly(A)+ RNA followed by antisense RNA synthesis in an in vitro transcription reaction with biotinylated or fluor-tagged nucleotides. The RNA probe is then fragmented to facilitate hybridization. If the indirect visualization method is used, the chips are incubated with fluor-linked streptavidin (e.g., phycoerythrin) after hybridization [12,33]. The signal is detected with a custom confocal scanner [34]. This method has been applied successfully to the mapping of genomic library clones [35], to de novo sequencing by hybridization [28,36], and to evolutionary sequence comparison of the *BRCA1* gene [37]. In addition, mutations in the cystic fibrosis [38] and BRCA1 [39] gene products and polymorphisms in the human immunodeficiency virus-1 clade B protease gene [40] have been detected by this method. Oligonucleotide chips are also useful for expression monitoring [33] as has been demonstrated by the simultaneous evaluation of gene-expression patterns in nearly all open reading frames of the yeast strain *S. cerevisiae* [12]. More recently, oligonucleotide chips have been used to help identify single nucleotide polymorphisms in the human [41] and yeast [42] genomes.

THE USE OF MICROARRAYS IN TOXICOLOGY

Screening for Mechanism of Action

The field of toxicology uses numerous in vivo model systems, including the rat, mouse, and rabbit, to assess potential toxicity and these bioassays are the mainstay of toxicology testing. However, in the past several decades, a plethora of in vitro techniques have been developed to measure toxicity, many of which measure toxicant-induced DNA damage. Examples of these assays include the Ames test, the Syrian hamster embryo cell transformation assay, micronucleus assays, measurements of sister chromatid exchange and unscheduled DNA synthesis, and many others. Fundamental to all of these methods is the fact that toxicity is often preceded by, and results in, alterations in gene expression. In many cases, these changes in gene expression are a

far more sensitive, characteristic, and measurable endpoint than the toxicity itself. We therefore propose that a method based on measurements of the genome-wide gene expression pattern of an organism after toxicant exposure is fundamentally informative and complements the established methods described above.

We are developing a method by which toxicants can be identified and their putative mechanisms of action determined by using toxicant-induced gene expression profiles. In this method, in one or more defined model systems, dose and time-course parameters are established for a series of toxicants within a given prototypic class (e.g., polycyclic aromatic hydrocarbons (PAHs)). Cells are then treated with these agents at a fixed toxicity level (as measured by cell survival), RNA is harvested, and toxicant-induced gene expression changes are assessed by hybridization to a cDNA microarray chip (Figure 1). We have developed a custom DNA chip, called ToxChip v1.0, specifically for this purpose and will discuss it in more detail below. The changes in gene expression induced by the test agents in the model systems are analyzed, and the common set of changes unique to that class of toxicants, termed a toxicant signature, is determined.

This signature is derived by ranking across all experiments the gene-expression data based on rela-

tive fold induction or suppression of genes in treated samples versus untreated controls and selecting the most consistently different signals across the sample set. A different signature may be established for each prototypic toxicant class. Once the signatures are determined, gene-expression profiles induced by unknown agents in these same model systems can then be compared with the established signatures. A match assigns a putative mechanism of action to the test compound. Figure 2 illustrates this signature method for different types of oxidant stressors, PAHs, and peroxisome proliferators. In this example, the unknown compound in question had a gene-expression profile similar to that of the oxidant stressors in the database. We anticipate that this general method will also reveal cross talk between different pathways induced by a single agent (e.g., reveal that a compound has both PAH-like and oxidant-like properties). In the future, it may be necessary to distinguish very subtle differences between compounds within a very large sample set (e.g., thousands of highly similar structural isomers in a combinatorial chemistry library or peptide library). To generate these highly refined signatures, standard statistical clustering techniques or principal-component analysis can be used.

For the studies outlined in Figure 2, we developed the custom cDNA microarray chip ToxChip v1.0.
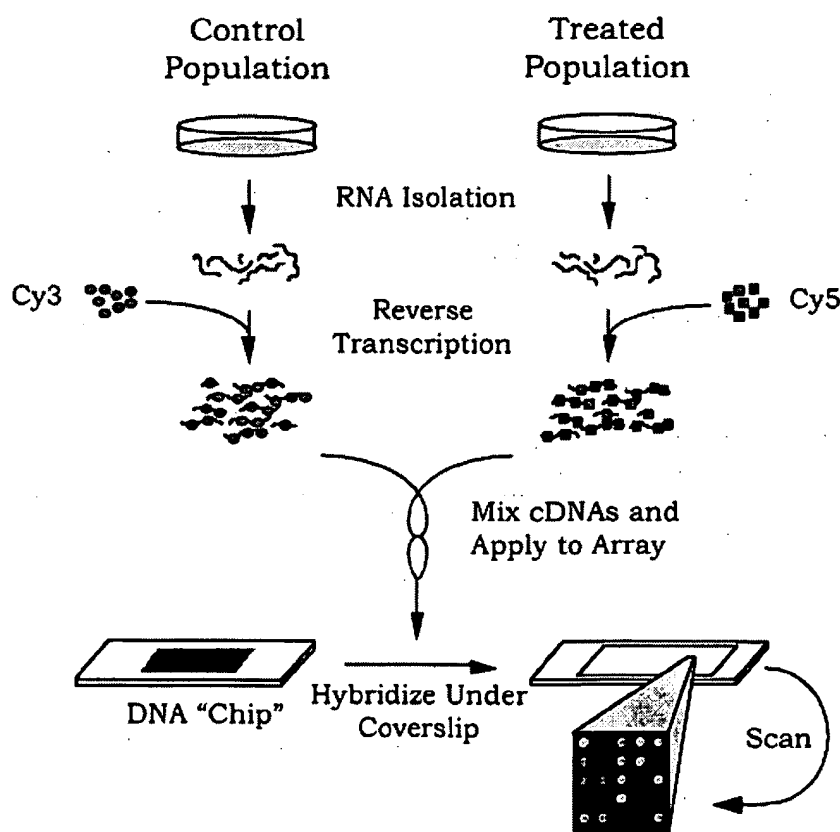
Figure 1. Simplified overview of the method for sample preparation and hybridization to cDNA microarrays. For illustrative purposes, samples derived from cell culture are depicted, although other sample types are amenable to this analysis.
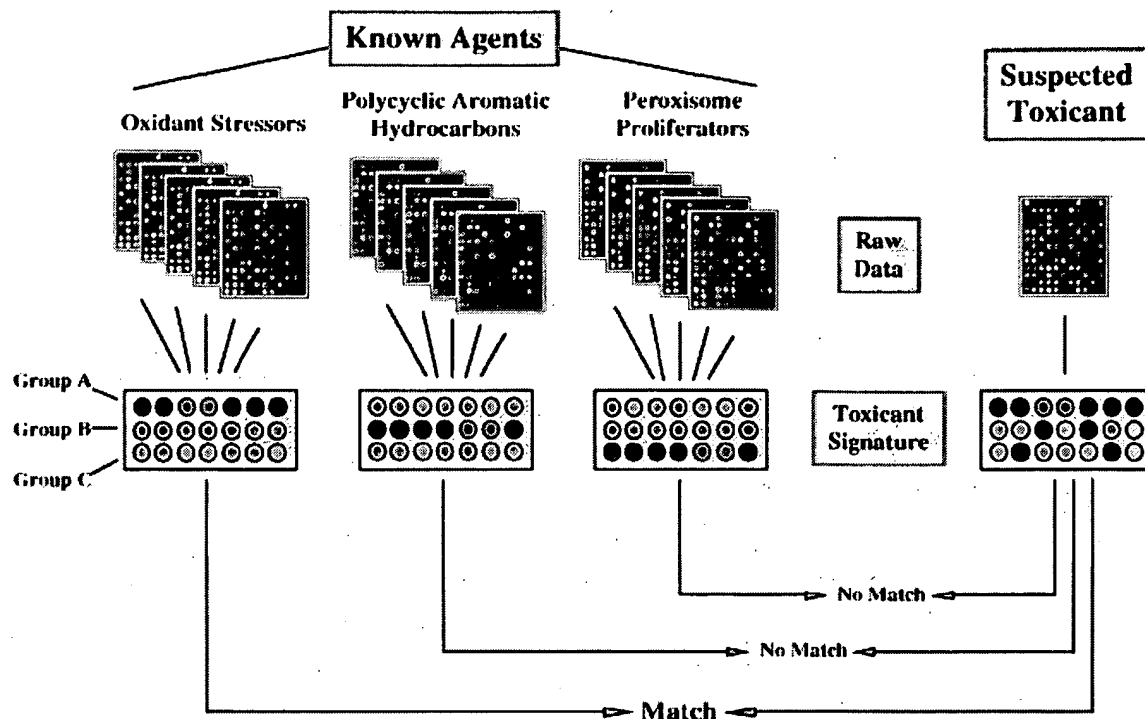
Figure 2. Schematic representation of the method for identification of a toxicant's mechanism of action. In this method, gene-expression data derived from exposure of model systems to known toxicants are analyzed, and a set of changes characteristic to that type of toxicant (termed the toxicant signature) is identified. As depicted, oxidant stressors produce consistent changes in group A genes (indicated by red and green circles), but not group B or C genes (indicated by gray circles). The set of gene-expression changes elicited by the suspected toxicant is then compared with these characteristic patterns, and a putative mechanism of action is assigned to the unknown agent.

The 2090 human genes that comprise this subarray were selected for their well-documented involvement in basic cellular processes as well as their responses to different types of toxic insult. Included on this list are DNA replication and repair genes, apoptosis genes, and genes responsive to PAHs and dioxin-like compounds, peroxisome proliferators, estrogenic compounds, and oxidant stress. Some of the other categories of genes include transcription factors, oncogenes, tumor suppressor genes, cyclins, kinases, phosphatases, cell adhesion and motility genes, and homeobox genes. Also included in this group are 84 housekeeping genes, whose hybridization intensity is averaged and used for signal normalization of the other genes on the chip. To date, very few toxicants have been shown to have appreciable effects on the expression of these housekeeping genes. However, this housekeeping list will be revised if new data warrant the addition or deletion of a particular gene. Table 1 contains a general description of some of the different classes of genes that comprise ToxChip v1.0.

When a toxicant signature is determined, the genes within this signature are flagged within the database. When uncharacterized toxicants are then screened, the data can be quickly reformatted so that blocks of genes representing the different signatures are displayed [11]. This facilitates rapid, visual interpretation of data. We are also developing Tox-Chip v2.0 and chips for other model systems, including rat, mouse, Xenopus, and yeast, for use in toxicology studies.

Animal Models in Toxicology Testing

The toxicology community relies heavily on the use of animals as model systems for toxicology testing. Unfortunately, these assays are inherently expensive, require large numbers of animals and take a long time to complete and analyze. Therefore, the National Institute of Environmental Health Sciences (NIEHS), the National Toxicology Program, and the toxicology community at large are committed to reducing the number of animals used, by developing more efficient and alternative testing methodologies. Although substantial progress has been made in the development of alternative methods, bioassays are still used for testing endpoints such as neurotoxicity, immunotoxicity, reproductive and developmental toxicology, and genetic toxicology. The rodent cancer bioassay is a particularly expensive and time-consuming assay, as it requires almost 4 yr, 1200 animals, and millions of dollars to execute and analyze [43]. In vitro experiments of the type outlined in Figure 2 might provide evidence that an unknown

Table 1. ToxChip v1.0: A Human cDNA Microarray Chip Designed to Detect Responses to Toxic Insult

Gene category	No. of genes on chip
Apoptosis	72
DNA replication and repair	99
Oxidative stress/redox homeostasis	90
Peroxisome proliferator responsive	22
Dioxin/PAH responsive	12
Estrogen responsive	63
Housekeeping	84
Oncogenes and tumor suppressor genes	76
Cell-cycle control	51
Transcription factors	131
Kinases	276
Phosphatases	88
Heat-shock proteins	23
Receptors	349
Cytochrome P450s	30

*This list is intended as a general guide. The gene categories are not unique, and some genes are listed in multiple categories.

agent is (or is not) responsible for eliciting a given biological response. This information would help to select a bioassay more specifically suited to the agent in question or perhaps suggest that a bioassay is not necessary, which would dramatically reduce cost, animal use, and time.

The addition of microarray techniques to standard bioassays may dramatically enhance the sensitivity and interpretability of the bioassay and possibly reduce its cost. Gene-expression signatures could be determined for various types of tissue-specific toxicants, and new compounds could be screened for these characteristic signatures, providing a rapid and sensitive in vivo test. Also, because gene expression is often exquisitely sensitive to low doses of a toxicant, the combination of gene-expression screening and the bioassay might allow the use of lower toxicant doses, which are more relevant to human exposure levels, and the use of fewer animals. In addition, gene-expression changes are normally measured in hours or days, not in the months to years required for tumor development. Furthermore, microarrays might be particularly useful for investigating the relationship between acute and chronic toxicity and identifying secondary effects of a given toxicant by studying the relationship between the duration of exposure to a toxicant and the gene-expression profile produced. Thus, a bioassay that incorporates gene-expression signatures with traditional endpoints might be substantially shorter, use more realistic dose regimens, and cost substantially less than the current assays do.

These considerations are also relevant for branches of toxicology not related to human health and not using rodents as model systems, such as aquatic toxicology and plant pathology. Bioassays based on the flathead minnow, *Daphnia,* and *Arabadopsis* could

also be improved by the addition of microarray analysis. The combination of microarrays with traditional bioassays might also be useful for investigating some of the more intractable problems in toxicology research, such as the effects of complex mixtures and the difficulties in cross-species extrapolation.

Exposure Assessment, Environmental Monitoring, and Drug Safety

The currently used methods for assessment of exposure to chemical toxicants are based on measurement of tissue toxin levels or on surrogate markers of toxicity, termed biomarkers (e.g., peripheral blood levels of hepatic enzymes or DNA adducts). Because gene expression is a sensitive endpoint, gene expression as measured with microarray technology may be useful as a new biomarker to more precisely identify hazards and to assess exposure. Similarly, microarrays could be used in an environmental-monitoring capacity to measure the effect of potential contaminants on the gene-expression profiles of resident organisms. In an analogous fashion, microarrays could be used to measure gene-expression endpoints in subjects in clinical trials. The combination of these gene-expression data and more established toxic endpoints in these trials could be used to define highly precise surrogates of safety.

Gene-expression profiles in samples from exposed individuals could be compared to the profiles of the same individuals before exposure. From this information, the nature of the toxic exposure can be determined or a relative clinical safety factor estimated. In the future it may also be possible to estimate not only the nature but the dose of the toxicant for a given exposure, based on relative gene-expression levels. This general approach may be particularly appropriate for occupational-health applications, in which unexposed and exposed samples from the same individuals may be obtainable. For example, a pilot study of gene expression in peripheral-blood lymphocytes of Polish coke-oven workers exposed to PAHs (and many other compounds) is under consideration at the NIEHS. An important consideration for these types of studies is that gene expression can be affected by numerous factors, including diet, health, and personal habits. To reduce the effects of these confounding factors, it may be necessary to compare pools of control samples with pools of treated samples. In the future it may be possible to compare exposed sample sets to a national database of human-expression data, thus eliminating the need to provide an unexposed sample from the same individual. Efforts to develop such a national gene-expression database are currently under way [44,45]. However, this national database approach will require a better understanding of genome-wide gene expression across the highly diverse human population and of the effects of environmental factors on this expression.

Alleles, Oligo Arrays, and Toxicogenetics

Gene sequences vary between individuals, and this variability can be a causative factor in human diseases of environmental origin [46,47]. A new area of toxicology, termed toxicogenetics, was recently developed to study the relationship between genetic variability and toxicant susceptibility. This field is not the subject of this discussion, but it is worthwhile to note that the ability of oligonucleotide arrays to discriminate DNA molecules based on single base-pair differences makes these arrays uniquely useful for this type of analysis. Recent reports demonstrated the feasibility of this approach [41,42]. The NIEHS has initiated the Environmental Genome Project to identify common sequence polymorphisms in 200 genes thought to be involved in environmental diseases [48]. In a pilot study on the feasibility of this application to the Environmental Genome Project, oligonucleotide arrays will be used to resequence 20 candidate genes. This toxicogenetic approach promises to dramatically improve our understanding of interindividual variability in disease susceptibility.

FUTURE PRIORITIES

There are many issues that must be addressed before the full potential of microarrays in toxicology research can be realized. Among these are model system selection, dose selection, and the temporal nature of gene expression. In other words, in which species, at what dose, and at what time do we look for toxicant-induced gene expression? If human samples are analyzed, how variable is global gene expression between individuals, before and after toxicant exposure? What are the effects of age, diet, and other factors on this expression? Experience, in the form of large data sets of toxicant exposures, will answer these questions.

One of the most pressing issues for array scientists is the construction of a national public database (linked to the existing public databases) to serve as a repository for gene-expression data. This relational database must be made available for public use, and researchers must be encouraged to submit their expression data so that others may view and query the information. Researchers at the National Institutes of Health have made laudable progress in developing the first generation of such a database [44,45]. In addition, improved statistical methods for gene clustering and pattern recognition are needed to analyze the data in such a public database.

The proliferation of different platforms and methods for microarray hybridizations will improve sample handling and data collection and analysis and reduce costs. However, the variety of microarray methods available will create problems of data compatibility between platforms. In addition, the near-infinite variety of experimental conditions under which data will be collected by different laboratories will make large-scale data analysis extremely difficult. To help circumvent these future problems, a set of standards to be included on all platforms should be established. These standards would facilitate data entry into the national database and serve as reference points for cross-platform and inter-laboratory data analysis.

Many issues remain to be resolved, but it is clear that new molecular techniques such as microarray hybridization will have a dramatic impact on toxicology research. In the future, the information gathered from microarray-based hybridization experiments will form the basis for an improved method to assess the impact of chemicals on human and environmental health.

ACKNOWLEDGMENTS

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Expression profiling in toxicology — potentials and limitations

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Abstract

Recent progress in genomics and proteomics technologies has created a unique opportunity to significantly impact the pharmaceutical drug development processes. The perception that cells and whole organisms express specific inducible responses to stimuli such as drug treatment implies that unique expression patterns, molecular fingerprints, indicative of a drug's efficacy and potential toxicity are accessible. The integration into state-of-the-art toxicology of assays allowing one to profile treatment-related changes in gene expression patterns promises new insights into mechanisms of drug action and toxicity. The benefits will be improved lead selection, and optimized monitoring of drug efficacy and safety in pre-clinical and clinical studies based on biologically relevant tissue and surrogate markers. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Proteomics; Genomics; Toxicology

1. Introduction

The majority of drugs act by binding to protein targets, most to known proteins representing enzymes, receptors and channels, resulting in effects such as enzyme inhibition and impairment of signal transduction. The treatment-induced perturbations provoke feedback reactions aiming to compensate for the stimulus, which almost always are associated with signals to the nucleus, resulting in altered gene expression. Such gene expression regulations account for both the pharmacological action and the toxicity of a drug and can be visualized by either global mRNA or global protein expression profiling. Hence, for each individual drug, a characteristic gene regulation pattern, its molecular fingerprint, exists which bears valuable information on its mode of action and its mechanism of toxicity.

Gene expression is a multistep process that results in an active protein (Fig. 1). There exist numerous regulation systems that exert control at and after the transcription and the translation step. Genomics, by definition, encompasses the quantitative analysis of transcripts at the mRNA level, while the aim of proteomics is to quantify gene expression further down-stream, creating a snapshot of gene regulation closer to ultimate cell function control.

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2. Global mRNA profiling

Expression data at the mRNA level can be produced using a set of different technologies such as DNA microarrays, reverse transcript imaging, amplified fragment length polymorphism (AFLP), serial analysis of gene expression (SAGE) and others. Currently, DNA microarrays are very popular and promise a great potential. On a typical array, each gene of interest is represented either by a long DNA fragment (200–2400 bp) typically generated by polymerase chain reaction (PCR) and spotted on a suitable substrate using robotics (Schena et al., 1995; Shalon et al., 1996) or by several short oligonucleotides (20–30 bp) synthesized directly onto a solid support using photolabile nucleotide chemistry (Fodor et al., 1991; Chee et al., 1996). From control and treated tissues, total RNA or mRNA is isolated and reverse transcribed in the presence of radioactive or fluorescent labeled nucleotides, and the labeled probes are then hybridized to the arrays. The intensity of the array signal is measured for each gene transcript by either autoradiography or laser scanning confocal microscopy. The ratio between the signals of control and treated samples reflect the relative drug-induced change in transcript abundance.

3. Global protein profiling

Global quantitative expression analysis at the protein level is currently restricted to the use of two-dimensional gel electrophoresis. This technique combines separation of tissue proteins by isoelectric focusing in the first dimension and by sodium dodecyl sulfate slab gel electrophoresis-based molecular weight separation on the second, orthogonal dimension (Anderson et al., 1991). The product is a rectangular pattern of protein spots that are typically revealed by Coomassie Blue, silver or fluorescent staining (Fig. 2). Protein spots are identified by mass spectrometry following generation of peptide mass fingerprints (Mann et al., 1993) and sequence tags (Wilkins et al., 1996). Similar to the mRNA approach, the ratio between the optical density of spots from control and treated samples are compared to search for treatment-related changes.

4. Expression data analysis

Bioinformatics forms a key element required to organize, analyze and store expression data from either source, the mRNA or the protein level. The overall objective, once a mass of high-quality
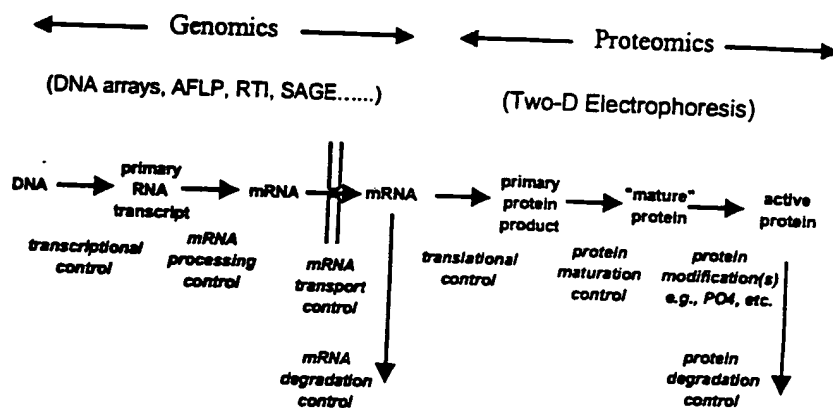
Fig. 1. Production of an active protein is a multistep process in which numerous regulation systems exert control at various stages of expression. Molecular fingerprints of drugs can be visualized through expression profiling at the mRNA level (genomics) using a variety of technologies and at the protein level (proteomics) using two-dimensional gel electrophoresis.
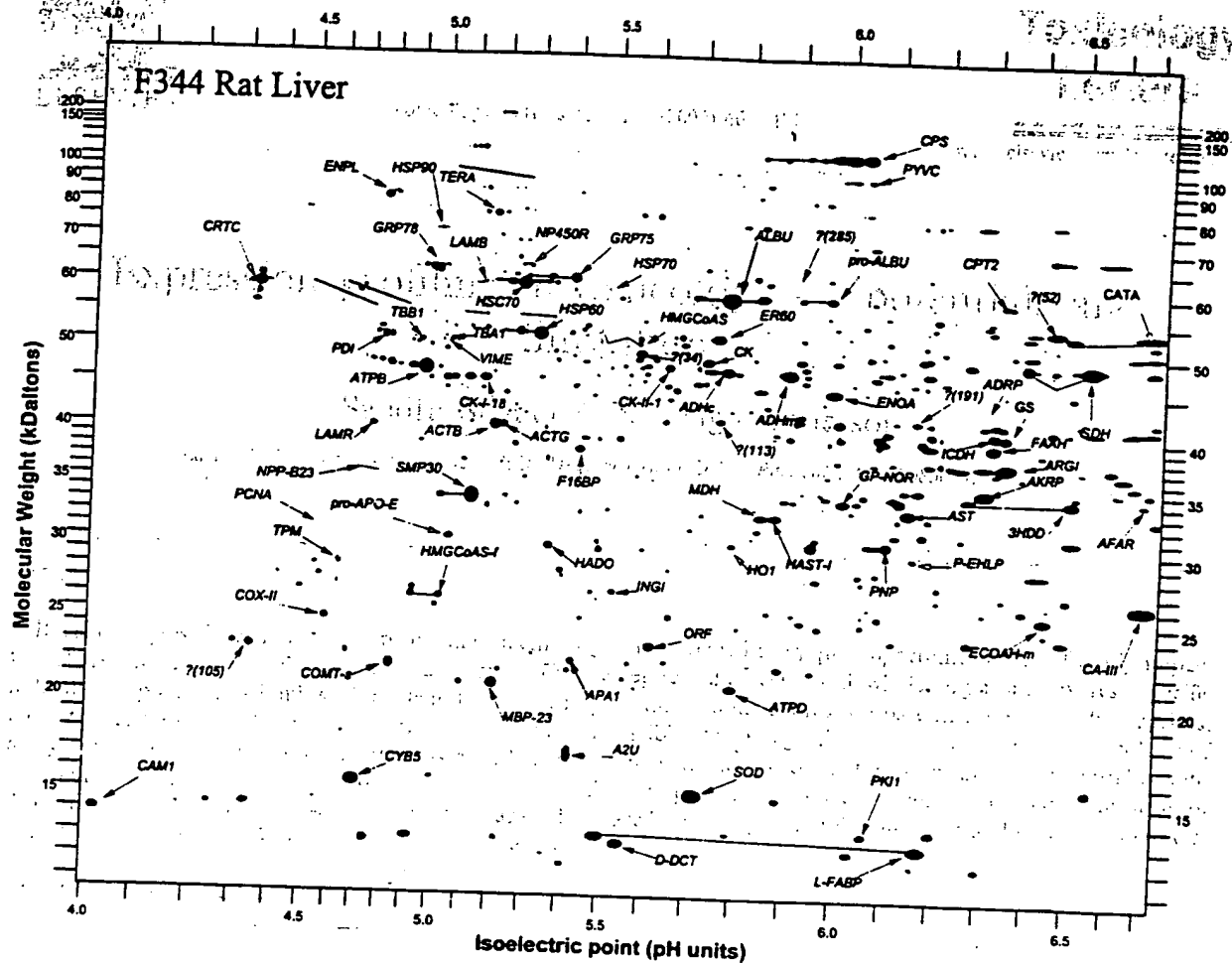
Fig. 2. Computerized representation of a Coomassie Blue stained two-dimensional gel electrophoresis pattern of Fischer F344 rat liver homogenate.

quantitative expression data has been collected, is to visualize complex patterns of gene expression changes, to detect pathways and sets of genes tightly correlated with treatment efficacy and toxicity, and to compare the effects of different sets of treatment (Anderson et al., 1996). As the drug effect database is growing, one may detect similarities and differences between the molecular fingerprints produced by various drugs, information that may be crucial to make a decision whether to refocus or extend the therapeutic spectrum of a drug candidate.

5. Comparison of global mRNA and protein expression profiling

There are several synergies and overlaps of data obtained by mRNA and protein expression analysis. Low abundant transcripts may not be easily quantified at the protein level using standard two-dimensional gel electrophoresis analysis and their detection may require prefractionation of samples. The expression of such genes may be preferably quantified at the mRNA level using techniques allowing PCR-mediated target amplifi-

cation. Tissue biopsy samples typically yield good quality of both mRNA and proteins; however, the quality of mRNA isolated from body fluids is often poor due to the faster degradation of mRNA when compared with proteins. RNA samples from body fluids such as serum or urine are often not very 'meaningful', and secreted proteins are likely more reliable surrogate markers for treatment efficacy and safety. Detection of post-translational modifications, events often related to function or nonfunction of a protein, is restricted to protein expression analysis and rarely can be predicted by mRNA profiling. Information on subcellular localization and translocation of proteins has to be acquired at the level of the protein in combination with sample prefractionation procedures. The growing evidence of a poor correlation between mRNA and protein abundance (Anderson and Seilhamer, 1997) further suggests that the two approaches, mRNA and protein profiling, are complementary and should be applied in parallel.

6. Expression profiling and drug development

Understanding the mechanisms of action and toxicity, and being able to monitor treatment efficacy and safety during trials is crucial for the successful development of a drug. Mechanistic insights are essential for the interpretation of drug effects and enhance the chances of recognizing potential species specificities contributing to an improved risk profile in humans (Richardson et al., 1993; Steiner et al. 1996b; Aicher et al., 1998). The value of expression profiling further increases when links between treatment-induced expression profiles and specific pharmacological and toxic endpoints are established (Anderson et al., 1991, 1995, 1996; Steiner et al. 1996a). Changes in gene expression are known to precede the manifestation of morphological alterations, giving expression profiling a great potential for early compound screening, enabling one to select drug candidates with wide therapeutic windows reflected by molecular fingerprints indicative of high pharmacological potency and low toxicity (Arce et al., 1998). In later phases of drug devel-

opment, surrogate markers of treatment efficacy and toxicity can be applied to optimize the monitoring of pre-clinical and clinical studies (Doherty et al., 1998).

7. Perspectives

The basic methodology of safety evaluation has changed little during the past decades. Toxicity in laboratory animals has been evaluated primarily by using hematological, clinical chemistry and histological parameters as indicators of organ damage. The rapid progress in genomics and proteomics technologies creates a unique opportunity to dramatically improve the predictive power of safety assessment and to accelerate the drug development process. Application of gene and protein expression profiling promises to improve lead selection, resulting in the development of drug candidates with higher efficacy and lower toxicity. The identification of biologically relevant surrogate markers correlated with treatment efficacy and safety bears a great potential to optimize the monitoring of pre-clinical and clinical trails.

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Application of DNA Arrays to Toxicology

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DNA array technology makes it possible to rapidly genotype individuals or quantify the expression of thousands of genes on a single filter or glass slide, and holds enormous potential in toxicologic applications. This potential led to a U.S. Environmental Protection Agency-sponsored workshop titled "Application of Microarrays to Toxicology" on 7–8 January 1999 in Research Triangle Park, North Carolina. In addition to providing state-of-the-art information on the application of DNA or gene microarrays, the workshop catalyzed the formation of several collaborations, committees, and user's groups throughout the Research Triangle Park area and beyond. Potential application of microarrays to toxicologic research and risk assessment include genome-wide expression analyses to identify gene-expression networks and toxicant-specific signatures that can be used to define mode of action, for exposure assessment, and for environmental monitoring. Arrays may also prove useful for monitoring genetic variability and its relationship to toxicant susceptibility in human populations. *Key words:* DNA arrays, gene arrays, microarrays, toxicology. *Environ Health Perspect* 107:681–685 (1999). [Online 6 July 1999]
http://ehpnet1.niehs.nih.gov/docs/1999/107p681-685rockett/abstract.html

Decoding the genetic blueprint is a dream that offers manifold returns in terms of understanding how organisms develop and function in an often hostile environment. With the rapid advances in molecular biology over the last 30 years, the dream has come a step closer to reality. Molecular biologists now have the ability to elucidate the composition of any genome. Indeed, almost 20 genomes have already been sequenced and more than 60 are currently under way. Foremost among these is the Human Genome Mapping Project. However, the genomes of a number of commonly used laboratory species are also under intensive investigation, including yeast, *Arabidopsis*, maize, rice, zebra fish, mouse, rat, and dog. It is widely expected that the completion of such programs will facilitate the development of many powerful new techniques and approaches to diagnosing and treating genetically and environmentally induced diseases which afflict mankind. However, the vast amount of data being generated by genome mapping will require new high-throughput technologies to investigate the function of the millions of new genes that are being reported. Among the most widely heralded of the new functional genomics technologies are DNA arrays, which represent perhaps the most anticipated new molecular biology technique since polymerase chain reaction (PCR).

Arrays enable the study of literally thousands of genes in a single experiment. The potential importance of arrays is enormous and has been highlighted by the recent publication of an entire *Nature Genetics* supplement dedicated to the technology (*1*). Despite this huge surge of interest, DNA arrays are still little used and largely unproven, as demonstrated by the high ratio of review and press articles to actual data papers. Even so, the potential they offer

has driven venture capitalists into a frenzy of investment and many new companies are springing up to claim a share of this rapidly developing market.

The U.S. Environmental Protection Agency (EPA) is interested in applying DNA array technology to ongoing toxicologic studies. To learn more about the current state of the technology, the Reproductive Toxicology Division (RTD) of the National Health and Environmental Effects Research Laboratory (NHEERL; Research Triangle Park, NC) hosted a workshop on "Application of Microarrays to Toxicology" on 7–8 January 1999 in Research Triangle Park, North Carolina. The workshop was organized by David Dix, Robert Kavlock, and John Rockett of the RTD/NHEERL. Twenty-two intramural and extramural scientists from government, academia, and industry shared information, data, and opinions on the current and future applications for this exciting new technology. The workshop had more than 150 attendees, including researchers, students, and administrators from the EPA, the National Institute of Environmental Health Sciences (NIEHS), and a number of other establishments from Research Triangle Park and beyond. Presentations ranged from the technology behind array production through the sharing of actual experimental data and projections on the future importance and applications of arrays. The information contained in the workshop presentations should provide aid and insight into arrays in general and their application to toxicology in particular.

Array Elements

In the context of molecular biology, the word "array" is normally used to refer to a series of DNA or protein elements firmly attached in

a regular pattern to some kind of supportive medium. DNA array is often used interchangeably with gene array or microarray. Although not formally defined, microarray is generally used to describe the higher density arrays typically printed on glass chips. The DNA elements that make up DNA arrays can be oligonucleotides, partial gene sequences, or full-length cDNAs. Companies offering pre-made arrays that contain less than full-length clones normally use regions of the genes which are specific to that gene to prevent false positives arising through cross-hybridization. Sequence verification of cDNA clone identity is necessary because of errors in identifying specific clones from cDNA libraries and databases. Premade DNA arrays printed on membranes are currently or imminently available for human, mouse, and rat. In most cases they contain DNA sequences representing several thousand different sequence clusters or genes as delineated through the National Center for Biotechnology Information UniGene Project (*2*). Many of these different UniGene clusters (putative genes) are represented only by expressed sequence tags (ESTs).

Array Printing

Arrays are typically printed on one of two types of support matrix. Nylon membranes are used by most off-the-shelf array providers such as Clontech Laboratories, Inc. (Palo Alto, CA), Genome Systems, Inc. (St. Louis, MO), and Research Genetics, Inc. (Huntsville, AL). Microarrays such as those produced by Affymetrix, Inc. (Santa Clara, CA), Incyte Pharmaceuticals, Inc. (Palo Alto, CA), and many do-it-yourself (DIY) arraying groups use glass wafers or slides. Although standard microscope slides may be used, they must be preprepared to facilitate sticking of the DNA to the glass. Several different

coatings have been successfully used, including silane and lysine. The coating of slides can easily be carried out in the laboratory, but many prefer the convenience of precoated slides available from suppliers.

Once the support matrix has been prepared, the DNA elements can be applied by several methods. Affymetrix, Inc., has developed a unique photolithographic technology for attaching oligonucleotides to glass wafers. More commonly, DNA is applied by either noncontact or contact printing. Noncontact printers can use thermal, solenoid, or piezoelectric technology to spray aliquots of solution onto the support matrix and may be used to produce slide or membrane-based arrays. Cartesian Technologies, Inc. (Irvine, CA) has developed nQUAD technology for use in its PixSys printers. The system couples a syringe pump with the microsolenoid valve, a combination that provides rapid quantitative dispensing of nanoliter volumes (down to 4.2 nL) over a variable volume range. A different approach to noncontact printing uses a solid pin and ring combination (Genetic MicroSystems, Inc., Woburn, MA). This system (Figure 1) allows a broader range of sample, including cell suspensions and particulates, because the printing head cannot be blocked up in the same way as a spray nozzle. Fluid transfer is controlled in this system primarily by the pin dimensions and the force of deposition, although the nature of the support matrix and the sample will also affect transfer to some degree.

In contact printing, the pin head is dipped in the sample and then touched to the support matrix to deposit a small aliquot. Split pins were one of the first contact-printing devices to be reported and are the suggested format for DIY arrayers, as described by Brown (3). Split pins are small metal pins with a precise groove cut vertically in the middle of the pin tip. In this system, 1–48 split pins are positioned in the pin-head. The split pins work by simple capillary action, not unlike a fountain pen—when the pin heads are dipped in the sample, liquid is drawn into the pin groove. A small (fixed) volume is then deposited each time the split pins are gently touched to the support matrix. Sample (100–500 pL depending on a variety of parameters) can be deposited on multiple slides before refilling is required, and array densities of > 2,500 spots/cm^2 may be produced. The deposit volume depends on the split size, sample fluidity, and the speed of printing. Split pins are relatively simple to produce and can be made in-house if a suitable machine shop is available. Alternatively, they can be obtained directly from companies such as TeleChem International, Inc. (Sunnyvale, CA).

Irrespective of their source, printers should be run through a preprint sequence prior to producing the actual experimental arrays; the first 100 or so spots of a new run tend to be somewhat variable. Factors effecting spot reproducibility include slide treatment homogeneity, sample differences, and instrument errors. Other factors that come into play include clean ejection of the drop and clogging (nQUAD printing) and mechanical variations and long-term alteration in print-head surface of solid and split pins. However, with careful preparation it is possible to get a coefficient of variance for spot reproducibility below 10%.

One potential printing problem is sample carryover. Repeated washing, blotting, and drying (vacuum) of print pins between samples is normally effective at reducing sample carryover to negligible amounts. Printing should also be carried out in a controlled environment. Humidified chambers are available in which to place printers. These help prevent dust contamination and produce a uniform drying rate, which is important in determining spot size, quality, and reproducibility.

In summary, although several printing technologies are available, none are particularly outstanding and the bottom line is that they are still in a relatively early stage of evolution.

Array Hybridization

The hybridization protocol is, practically speaking, relatively straightforward and those with previous experience in blotting should have little difficulty. Array hybridizations are, in essence, reverse Southern/Northern blots—instead of applying a labeled probe to the target population of DNA/RNA, the labeled population is applied to the probe(s). With membrane-based arrays, the control and treated mRNA populations are normally converted to cDNA and labeled with isotope (e.g., ^{33}P) in the process. These labeled populations are then hybridized independently to parallel or serial arrays and the hybridization signal is detected with a phosporimager. A less commonly used alternative to radioactive probes is enzymatic detection. The probe may be biotinylated, haptenylated, or have alkaline phosphatase/horseradish peroxidase attached. Hybridization is detected by enzymatic reaction yielding a color reaction (4). Differences in hybridization signals can be detected by eye or, more accurately, with the help of digital imaging and commercially available software. The labeling of the test populations for slide-based microarrays uses a slightly different approach. The probe typically consists of two samples of polyA$^+$ RNA (usually from a treated and a control population) that are converted to cDNA; in the process each is labeled with a different fluor. The independently labeled probes are then mixed together and hybridized to a single microarray slide and the resulting combined fluorescent signal is scanned. After
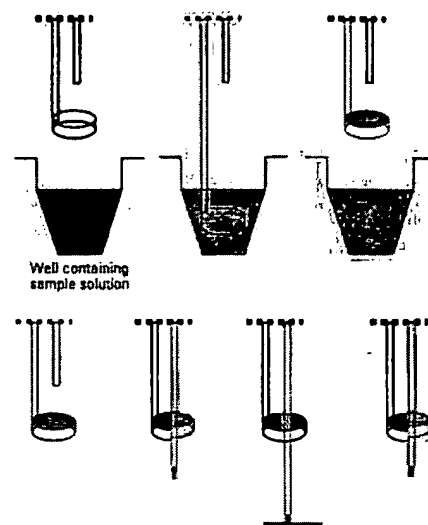
Figure 1. Genetic Microsystems (Woburn, MA) pin ring system for printing arrays. The pin ring combination consists of a circular open ring oriented parallel to the sample solution, with a vertical pin centered over the ring. When the ring is dipped into a solution and lifted, it withdraws an aliquot of sample held by surface tension. To spot the sample, the pin is driven down through the ring and a portion of the solution is transferred to the bottom of the pin. The pin continues to move downward until the pendant drop of solution makes contact with the underlying surface. The pin is then lifted, and gravity and surface tension cause deposition of the spot onto the array. Figure from Flowers et al. (14), with permission from Genetic Microsystems.

normalization, it is possible to determine the ratio of fluorescent signals from a single hybridization of a slide-based microarray.

cDNA derived from control and treated populations of RNA is most commonly hybridized to arrays, although subtractive hybridization or differential display reactions may also be used. Fluorophore- or radiolabeled nucleotides are directly incorporated into the cDNA in the process of converting RNA to cDNA. Alternatively, 5' end-labeled primers may be used for cDNA synthesis. These are labeled with a fluorophore for direct visualization of the hybridized array. Alternatively, biotin or a hapten may be attached to the primer, in which case fluorlabeled streptavidin or antibody must be applied before a signal can be generated. The most commonly used fluorophores at present are cyanine (Cy)3 and Cy5 (Amersham Pharmacia Biotech AB, Uppsala, Sweden). However, the relative expense of these fluorescent conjugates has driven a search for cheaper alternatives. Fluorescein, rhodamine, and Texas red have all been used, and companies such as Molecular Probes, Inc. (Eugene, OR) are developing a series of labeled nucleotides with a wide range of excitation and emission spectra which may prove to function as well as the Cy dyes.

Analysis of DNA Microarrays

Membrane-based arrays are normally analyzed on film or with a phosphorimager, whereas chip-based arrays require more specialized scanning devices. These can be divided into three main groups: the charge-coupled device camera systems, the nonconfocal laser scanners, and the confocal laser scanners. The advantages and disadvantages of each system are listed in Table 1.

Because a typical spot on a microarray can contain > 10^8 molecules, it is clear that a large variation in signal strength may occur. Current scanners cannot work across this many orders of magnitude (4 or 5 is more typical). However, the scanning parameters can normally be adjusted to collect more or less signal, such that two or three scans of the same array should permit the detection of rare and abundant genes.

When a microarray is scanned, the fluorescent images are captured by software normally included with the scanner. Several commercial suppliers provide additional software for quantifying array images, but the software tools are constantly evolving to meet the developing needs of researchers, and it is prudent to define one's own needs and clarify the exact capabilities of the software before its purchase. Issues that should be considered include the following:

- Can the software locate offset spots?
- Can it quantitate across irregular hybridization signals?
- Can the arrayed genes be programmed in for easy identification and location?
- Can the software connect via the Internet to databases containing further information on the gene(s) of interest?

One of the key issues raised at the workshop was the sensitivity of microarray technology. Experiments by General Scanning, Inc. (Watertown, MA), have shown that by using the Cy dyes and their scanner, signal can be detected down to levels of < 1 fluor molecule per square micrometer, which translates to detecting a rare message at approximately one copy per cell or less.

Array Applications

Although arrays are an emerging technology certain to undergo improvement and alteration, they have already been applied usefully to a number of model systems. Arrays are at their most powerful when they contain the entire genome of the species they are being used to study. For this reason, they have strong support among researchers utilizing yeast and *Caenorhabditis elegans* (5). The genomes of both of these species have been sequenced and, in the case of yeast, deposited onto arrays for examination of gene expression (6,7). With both of these species, it is relatively easy to perturb individual gene expression. Indeed, *C.*

elegans knockouts can be made simply by soaking the worms in an antisense solution of the gene to be knocked out.

By a process of systematic gene disruption, it is now possible to examine the cause and effect relationships between different genes in these simple organisms. This kind of approach should help elucidate biochemical pathways and genetic control processes, deconvolute polygenic interactions, and define the architecture of the cellular network. A simple case study of how this can be achieved was presented by Butow [University of Texas Southwestern Medical Center, Dallas, TX (Figure 2)]. Although it is the phenotypic result of a single gene knockout that is being examined, the effect of such perturbation will almost always be polygenic. Polygenic interactions will become increasingly important as researchers begin to move away from single gene systems when examining the nature of toxicologic responses to external stimuli. This is especially important in toxicology because the phenotype produced by a given environmental insult is never the result of the action of a single gene; rather, it is a complex interaction of one or multiple cellular pathways. Phenomena such as quantitative trait (the continuous variation of phenotype), epistasis (the effect of alleles of one or more genes on the expression of other genes), and penetrance (proportion of individuals of a given genotype that display a particular phenotype) will become increasingly evident and important as toxicologists push toward the ultimate goal of matching the responses of individuals to different environmental stimuli.

Analysis of the transcriptome (the expression level of all the genes in a given cell population) was a use of arrays addressed by several speakers. Unfortunately, current gene nomenclature is often confusing in that single genes are allocated multiple names (usually as a result of independent discovery by different laboratories), and there was a call for standardization of gene nomenclature. Nevertheless, once a transcriptome has been assembled it can then be transferred onto arrays and used to screen any chosen system. The EPA MicroArray Consortium (EPAMAC) is assembling testes

transcriptomes for human, rat, and mouse. In a slightly different approach, Nuwaysir et al. (8) describes how the NIEHS assembled what is effectively a "toxicological transcriptome"—a library of human and mouse genes that have previously been proven or implicated in responses to toxicologic insults. Clontech Laboratories, Inc. (Palo Alto, CA), has begun a similar process by developing stress/toxicology filter arrays of rat, mouse, and human genes. Thus, rather than being tissue or cell specific, these stress/toxicology arrays can be used across a variety of model systems to look for alterations in the expression of toxicologically important genes and define the new field of toxicogenomics. The potential to identify toxicant families based on tissue- or cell-specific gene expression could revolutionize drug testing. These molecular signatures or fingerprints could not only point to the possible toxicity/carcinogenicity of newly discovered compounds (Figure 3), but also aid in elucidating their mechanism of action through identification of gene expression networks. By extension, such signatures could provide easily identifiable biomarkers to assess the degree, time, and nature of exposure.

DNA arrays are primarily a tool for examining differential gene expression in a given model. In this context they are referred to as closed systems because they lack the ability of other differential expression technologies, e.g., differential display and subtractive hybridization, to detect previously unknown genes not present on the array. This would appear to limit the power of DNA arrays to the imaginations and preconceptions of the researcher in selecting genes previously characterized and thought to be involved in the model system. However, the various genome sequencing projects have created a new category of sequence—the EST—that has partially mollified this deficiency. ESTs are cDNAs expressed in a given tissue that, although they may share some degree of sequence similarity to previously characterized genes, have not been assigned specific genetic identity. By incorporating EST clones into an array, it is possible to monitor the expression of these unknown genes. This can enable the identification of previously uncharacterized genes that may have biologic

Table 1. Advantages and disadvantages of different microarray scanning systems.

	Nonconfocal laser scanner		
Advantages	Few moving parts	Relatively simple optics	Small depth of focus reduces artifacts
	Fast scanning of bright samples		May have high light collection efficiency
Disadvantages	Less appropriate for dim samples	Low light collection efficiency	Small depth of focus requires scanning precision
	Optical scatter can limit performance	Background artifacts not rejected	
		Resolution typically low	

CCD, charge-coupled device.
From Kawasaki (13).

significance in the model system. Filter arrays from Research Genetics and slide arrays from Incyte Pharmaceuticals both incorporate large numbers of ESTs from a variety of species.

A further use of microarrays is the identification of single nucleotide polymorphisms (SNPs). These genomic variations are abundant—they occur approximately every 1 kb or so—and are the basis of restriction fragment length polymorphism analysis used in forensic analysis. Affymetrix, Inc., designed chips that contain multiple repeats of the same gene sequence. Each position is present with all four possible bases. After the hybridization of the sample, the degree of hybridization to the different sequences can be measured and the exact sequence of the target gene deduced. SNPs are thought to be of vital importance in drug metabolism and toxicology. For example, single base differences in the regulatory region or active site of some genes can account for huge differences in the activity of that gene. Such SNPs are thought to explain why some people are able to metabolize certain xenobiotics better than others. Thus, arrays provide a further tool for the toxicologist investigating the nature of susceptible subpopulations and toxicologic response.

There are still many wrinkles to be ironed out before arrays become a standard tool for toxicologists. The main issues raised at the workshop by those with hands-on experience were the following:

• Expense: the cost of purchasing/contracting this technology is still too great for many individual laboratories.
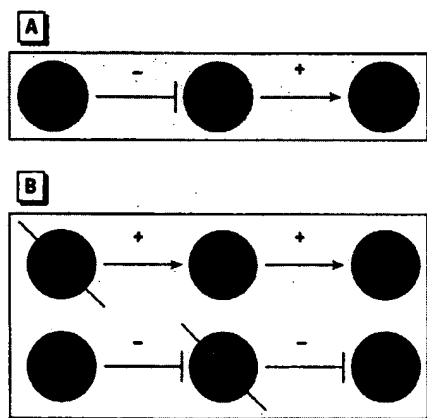
Figure 2. Potential effects of gene knockout within positively and negatively regulated gene expression networks. i_1 is limiting in wild type for expression of i_2. (*A*) A simple, two-component, linear regulatory network operating on gene i_2, where i_1 is a positive effector of i_2 and j_n is either a positive or negative effector of i_1. This network could be deduced by examining the consequence of (*B*) deleting j_n on the expression of i_1 and i_2, where the expression of i_2 would be decreased or increased depending on whether j_n was a positive or negative regulator. These and other connected components of even greater complexity could be revealed by genome-wide expression analysis. From Butow (*15*).

• Clones: the logistics of identifying, obtaining, and maintaining a set of nonredundant, non-contaminated, sequence-verified, species/cell/tissue/field-specific clones.
• Use of inbred strains: where whole-organism models are being used, the use of inbred strains is important to reduce the potentially confusing effects of the individual variation typically seen in outbred populations.
• Probe: the need for relatively large amounts of RNA, which limits the type of sample (e.g., biopsy) that can be used. Also, different RNA extraction methods can give different results.
• Specificity: the ability to discriminate accurately between closely related genes (e.g., the cytochrome p450 family) and splice variants.
• Quantitation: the quantitation of gene expression using gene arrays is still open to debate. One reason for this is the different incorporation of the labeling dyes. However, the main difficulty lies in knowing what to normalize against. One option is to include a large number of so-called housekeeping genes in the array. However, the expression of these genes often change depending on the tissue and the toxicant, so it is necessary to characterize the expression of these genes in the model system before utilizing them. This is clearly not a viable option when screening multiple new compounds. A second option is to include on the array genes from a nonrelated species (e.g., a plant gene on an animal array) and to spike the probe with synthetic RNA(s) complementary to the gene(s).
• Reproducibility: this is sometimes questionable, and a figure of approximately two or three repeats was used as the minimum number required to confirm initial findings.

Again, however, most people advocated the use of Northern blots or reverse transcriptase PCR to confirm findings.
• Sensitivity: concerns were voiced about the number of target molecules that must be present in a sample for them to be detected on the array.
• Efficiency: reproducible identification of 1.5- to 2-fold differences in expression was reported, although the number of genes that undergo this level of change and remain undetected is open to debate. It is important that this level of detection be ultimately achieved because it is commonly perceived that some important transcription factors and their regulators respond at such low levels. In most cases, 3- to 5-fold was the minimum change that most were happy to accept.
• Bioinformatics: perhaps the greatest concern was how to accurately interpret the data with the greatest accuracy and efficiency. The biggest headache is trying to identify networks of gene expression that are common to different treatments or doses. The amount of data from a single experiment is huge. It may be that, in the future, several groups individually equipped with specialized software algorithms for studying their favorite genes or gene systems will be able to share the same hybridized chips. Thus, arrays could usher in a new perspective on collaboration and the sharing of data.

EPAMAC

Perhaps the main reason most scientists are unable to use array technology is the high cost involved, whether buying off-the-shelf membranes, using contract printing services, or
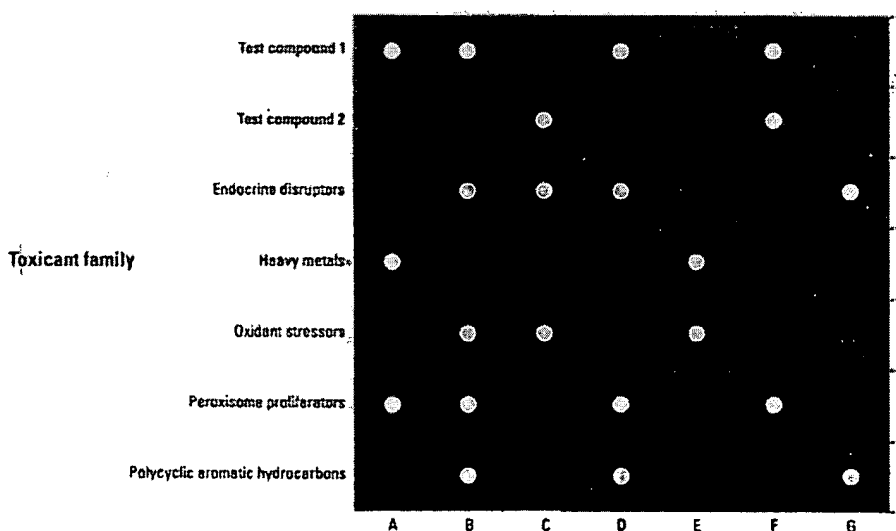
Figure 3. Gene expression profiles—also called fingerprints or signatures—of known toxicants or toxicant families may, in the future, be used to identify the potential toxicity of new drugs, etc. In this example, the genetic signature of test compound 1 is identical to that of known peroxisome proliferators, whereas that of test compound 2 does not match any known toxicant family. Based on these results, test compound 2 would be retained for further testing and test compound 1 would be eliminated.

producing chips in-house. In view of this, researchers at the RTD/NHEERL initiated the EPAMAC. This consortium brings together scientists from the EPA and a number of extramural labs with the aim of developing microarray capability through the sharing of resources and data. EPAMAC researchers are primarily interested in the developmental and toxicologic changes seen in testicular and breast tissue, and a portion of the workshop was set aside for EPAMAC members to share their ideas on how the experimental application of microarrays could facilitate their research. One of the central areas of interest to EPAMAC members is the effect of xenobiotics on male fertility and reproductive health. Of greatest concern is the effect of exposure during critical periods of development and germ cell differentiation (9), and how this may compromise sperm counts and quality following sexual maturation (10). As well as spermatogenic tissue, there is also interest in how residual mRNA found in mature sperm (11) could be used as an indicator of previous xenobiotic effects (it is easier to obtain a semen sample than a testicular biopsy). Arrays will be used to examine and compare the effect of exposure to heat and chemicals in testicular and epididymal gene expression profiles, with the aim of establishing relationships/associations between changes in developmental landmarks and the effects on sperm count and quality. Cluster, pattern, and other analysis of such data should help identify hidden relationships between genes that may reveal potential mechanisms of action and uncover roles for genes with unknown functions.

Summary

The full impact of DNA arrays may not be seen for several years, but the interest shown at this regional workshop indicates the high level of interest that they foster. Apart from educating and advertising the various technologies in this field, this workshop brought together a number of researchers from the Research Triangle Park area who are already using DNA arrays. The interest in sharing ideas and experiences led to the initiation of a Triangle array user's group.

Array technology is still in its infancy. This means that the hardware is still improving and there is no current consensus for standard procedures, quantitation, and interpretation. Consistency in spotting and scanning arrays is not yet optimized, and this is one of the most critical requirements of any experiment. In addition, one of the dark regions of array technology—strife in the courts over who owns what portions of it—has further muddled the future and is a potential barrier toward the development of consensus procedures.

Perhaps the greatest hurdle for the application of arrays is the actual interpretation of data. No specialists in bioinformatics attended the workshop, largely because they are rare and because as yet no one seems clear on the best method of approaching data analysis and interpretation. Cross-referencing results from multiple experiments (time, dose, repeats, different animals, different species) to identify commonly expressed genes is a great challenge. In most cases, we are still a long way from understanding how the expression of gene X is related to the expression of gene Y, and ordering gene expression to delineate causal relationships.

To the ordinary scientist in the typical laboratory, however, the most immediate problem is a lack of affordable instrumentation. One can purchase premade membranes at relatively affordable prices. Although these may be useful in identifying individual genes to pursue in more detail using other methods, the numbers that would be required for even a small routine toxicology experiment prohibit this as a truly viable approach. For the toxicologist, there is a need to carry out multiple experiments—dose responses, time curves, multiple animals, and repeats. Glass-based DNA arrays are most attractive in this context because they can be prepared in large batches from the same DNA source and accommodate control and treated samples on the same chip. Another problem with current off-the-shelf arrays is that they often do not contain one or more of the particular genes a group is interested in. One alternative is to obtain and/or produce a set of custom clones and have contract printing of membranes or slides carried out by a company such as Genomic Solutions, Inc. (Ann Arbor, MI). This approach

is less expensive than laying out capital for one's own entire system, although at some point it might make economic sense to print one's own arrays.

Finally, DNA arrays are currently a team effort. They are a technology that uses a wide range of skills including engineering, statistics, molecular biology, chemistry, and bioinformatics. Because most individuals are skilled in only one or perhaps two of these areas, it appears that success with arrays may be best expected by teams of collaborators consisting of individuals having each of these skills.

Those considering array applications may be amused or goaded on by the following quote from *Fortune* magazine (12):

> Microprocessors have reshaped our economy, spawned vast fortunes and changed the way we live. Gene chips could be even bigger.

Although this comment may have been designed to excite the imagination rather than accurately reflect the truth, it is fair to say that the age of functional genomics is upon us. DNA arrays look set to be an important tool in this new age of biotechnology and will likely contribute answers to some of toxicology's most fundamental questions.

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SPEAKERS

Cindy Afshari	Abdel Elkahloun	Steve Krawetz	Jim Samet
NIEHS	Research Genetics, Inc.	Wayne State University	U.S. EPA
Linda Birnbaum	Sue Fenton	Nick Mace	Sam Ward
U.S. EPA	U.S. EPA	Genetic Microsystems, Inc.	University of Arizona
Ron Butow	Norman Hecht	Scott Mordecai	Jeff Welch
University of Texas	University of Pennsylvania	Affymetrix, Inc.	U.S. EPA
Southwestern Medical	Pat Hurban	Kevin Morgan	Reen Wu
Center	Paradigm Genetics, Inc.	Glaxo Wellcome, Inc.	University of California
Alex Chenchik	Bob Kavlock	Elaine Poplin	at Davis
Clontech Laboratories, Inc.	U.S. EPA	Research Genetics, Inc.	Tim Zacharewski
David Dix	Ernie Kawasaki	Don Rose	Michigan State University
U.S. EPA	General Scanning, Inc.	Cartesian Technologies, Inc.	

Subject: RE: [Fwd: Toxicology Chip]
Date: Mon. 3 Jul 2000 08:09:45 -0400
From: "Afshari.Cynthia" <afshari@niehs.nih.gov>
To: "Diana Hamlet-Cox" <dianahc@incyte.com>

You can see the list of clones that we have on our 12K chip at
http: manuel.niehs.nih.gov maps guest clonesrch.cfm
We selected a subset of genes (2000K) that we believed critical to tox
response and basic cellular processes and added a set of clones and ESTs to
this. We have included a set of control genes (80+) that were selected by
the NHGRI because they did not change across a large set of array
experiments. However, we have found that some of these genes change
significantly after tox treatments and are in the process of looking at the
variation of each of these 80+ genes across our experiments.
Our chips are constantly changing and being updated and we hope that our
data will lead us to what the toxchip should really be.
I hope this answers your question.
Cindy Afshari

> ----------
> From: Diana Hamlet-Cox
> Sent: Monday, June 26, 2000 8:52 PM
> To: afshari@niehs.nih.gov
> Subject: [Fwd: Toxicology Chip]
>
> Dear Dr. Afshari,
>
> Since I have not yet had a response from Bill Grigg, perhaps he was not
> the right person to contact.
>
> Can you help me in this matter? I don't need to know the sequences,
> necessarily, but I would like very much to know what types of sequences
> are being used, e.g., GPCRs (more specific?), ion channels, etc.
>
> Diana Hamlet-Cox
>
> --------- Original Message --------
> Subject: Toxicology Chip
> Date: Mon, 19 Jun 2000 18:31:48 -0700
> From: Diana Hamlet-Cox <dianahc@incyte.com>
> Organization: Incyte Pharmaceuticals
> To: grigg@niehs.nih.gov
>
> Dear Colleague:
>
> I am doing literature research on the use of expressed genes as
> pharmacotoxicology markers, and found the Press Release dated February
> 29, 2000 regarding the work of the NIEHS in this area. I would like to
> know if there is a resource I can access (or you could provide?) that
> would give me a list of the 12,000 genes that are on your Human ToxChip
> Microarray. In particular, I am interested in the criteria used to
> select sequences for the ToxChip, including any control sequences
> included in the microarray.
>
> Thank you for your assistance in this request.
>
> Diana Hamlet-Cox, Ph.D.
> Incyte Genomics, Inc.
>
> --
>
> ==========================

research focus   **REVIEWS**

Proteomics: a major new technology for the drug discovery process

Martin J. Page, Bob Amess, Christian Rohlff, Colin Stubberfield and Raj Parekh

Proteomics is a new enabling technology that is being integrated into the drug discovery process. This will facilitate the systematic analysis of proteins across any biological system or disease, forwarding new targets and information on mode of action, toxicology and surrogate markers. Proteomics is highly complementary to genomic approaches in the drug discovery process and, for the first time, offers scientists the ability to integrate information from the genome, expressed mRNAs, their respective proteins and subcellular localization. It is expected that this will lead to important new insights into disease mechanisms and improved drug discovery strategies to produce novel therapeutics.

Among the major pharmaceutical and biotechnology companies, it is clearly recognized that the business of modern drug discovery is a highly competitive process. All of the many steps involved are inherently complex, and each can involve a high risk of attrition. The players in this business strive continuously to optimize and streamline the process; each seeking to gain an advantage at every step by attempting to make informed decisions at the earliest stage possible. The desired outcome is to accelerate as many key activities in the drug discovery process as possible. This should produce a new generation of robust drugs that offer a high probability of success and reach the clinic and market ahead of the competition.

There has been noticeable emphasis over recent years for companies to aggressively review and refine their strategies to discover new drugs. Central to this has been the introduction and implementation of cutting-edge technologies. Most, if not all, companies have now integrated key technology platforms that incorporate genomics, mRNA expression analysis, relational databases, high-throughput robotics, combinatorial chemistry and powerful bioinformatics. Although it is still early days to quantify the real impact of these platforms in clinical and commercial terms, expectations are high, and it is widely accepted that significant benefits will be forthcoming. This is largely based on data obtained during preclinical studies where the genomic[1,2] and microarray[3,4] technologies have already proved their value.

However, there are several noteworthy outcomes that result from this. Many comments are voiced that scientists armed with these technologies are now commonly faced with data overload. Thus, in some instances, rather than facilitating the decision process, the accumulation of more complex data points, many with unknown consequences, can seem to hinder the process. Also, most drug companies have simultaneously incorporated very similar components of the new technology platforms, the consequence being that it is becoming difficult yet again to determine where a clear competitive advantage will arise. Finally, in recent years, largely as a result of the accessibility of the technologies, there has been an overwhelming emphasis placed on genomic and mRNA data rather than on protein

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Figure 1. Steps involved in analysing a biological sample by proteomics. MCI, molecular cluster index.
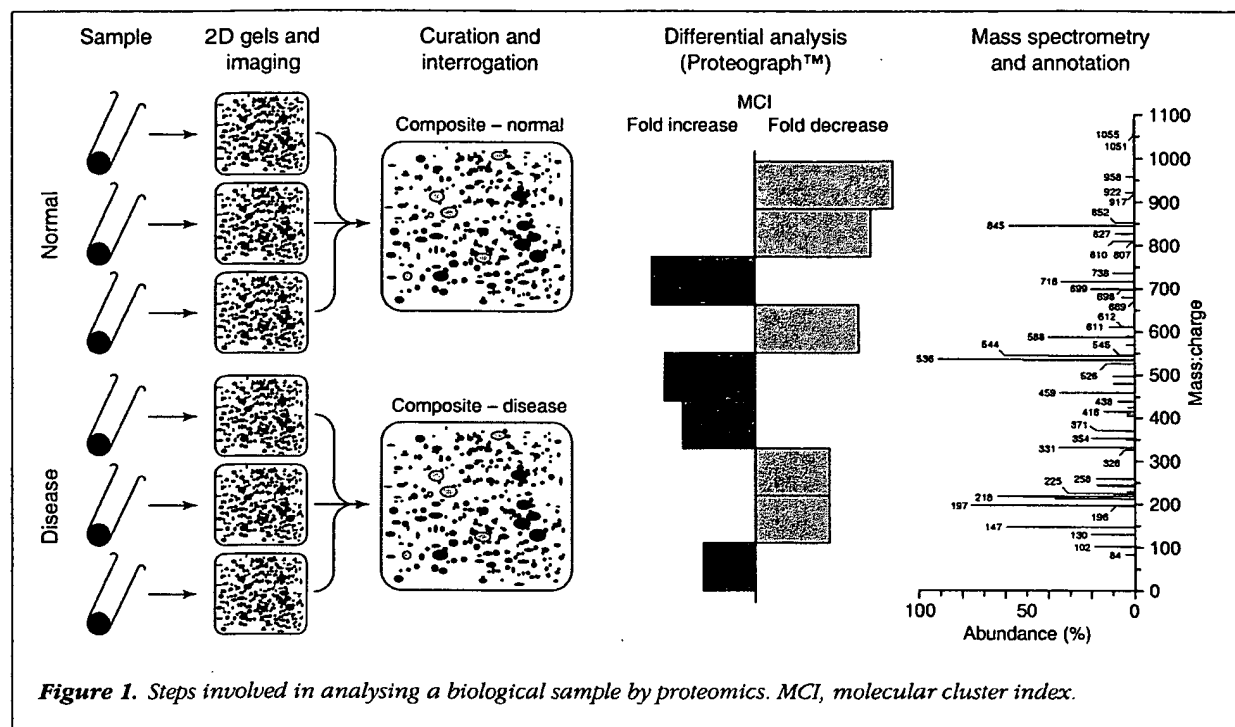
analysis. It is important to remember that proteins dictate biological phenotype – whether it is normal or diseased – and are the direct targets for most drugs.

Proteomics: new technology for the analysis of proteins

It is now timely to recognize that complementary technology in the form of high-throughput analysis of the total protein repertoire of chosen biological samples, namely proteomics, is poised to add a new and important dimension to drug discovery. In a similar fashion to genomics, which aims to profile every gene expressed in a cell, proteomics seeks to profile every protein that is expressed[5-7]. However, there is added information, since proteomics can also be used to identify the post-translational modifications of proteins[8], which can have profound effects on biological function, and their cellular localization. Importantly, proteomics is a technology that integrates the significant advances in two-dimensional (2D) electrophoretic separation of proteins, mass spectrometry and bioinformatics. With these advances it is now possible to consistently derive proteomes that are highly reproducible and suitable for interrogation using advanced bioinformatic tools.

There are many variations whereby different laboratories operate proteomics. For the purpose of this review, the process used at Oxford GlycoSciences (OGS), which uses an industrial-scale operation that is integral to its drug discovery work, will be described. The individual steps of this process, where up to 1000 2D gels can be run and analysed per week, are summarized in Fig. 1. The incoming samples are bar coded and all information relevant to the sample is logged into a Laboratory Information Management System (LIMS) database. There can be a wide range in the type of samples processed, as applicable to individual steps in the drug discovery pipeline, and these will be mentioned later. The samples are separated according to their charge (pI) in the first dimension, using isoelectric focusing, followed by size (MW) using SDS–PAGE in the second dimension. Many modifications have been made to these steps to improve handling, throughput and reproducibility. The separated proteins are then stained with fluorescent dyes which are significantly more sensitive in detection than standard silver methods and have a broader dynamic range. The image of the displayed proteins obtained is referred to as the proteome, and is digitally scanned into databases using proprietary software called ROSETTA™. The images are subsequently curated, which begins with the removal of any artefacts, cropping and the placement of pI/MW landmarks. The images from replicate images are then aligned and matched to one

another to generate a synthetic composite image. This is an important step, as the proteome is a dynamic situation, and it captures the biological variation that occurs, such that even orphan proteins are still incorporated into the analysis.

By means of illustration, Fig. 1 shows the process whereby proteomes are generated from normal and disease samples and how differentially expressed proteins are identified. The potential of this type of analysis is tremendous. For example, from a mammalian cell sample, in excess of 2000 proteins can typically be resolved within the proteome. The quality of this is shown in Fig. 2, which shows representative proteomes from three diverse biological sources: human serum, the pathogenic fungus *Candida albicans* and the human hepatoma cell line Huh7.

Use of proteomics to identify disease specific proteins

In most cases, the drug discovery process is initiated by the identification of a novel candidate target – almost always a protein – that is believed to be instrumental in the disease process. To date, there is a variety of means whereby drug targets have been forthcoming. These include molecular, cellular and genomic approaches, mostly centred upon DNA and mRNA analysis. The gene in question is isolated, and expression and characterization of its coded protein product – i.e. the drug target – is invariably a secondary event.

With the proteomic approach, the starting point is at the other end of the 'telescope'. Here there is direct and im-

mediate comparison of the proteomes from paired normal and disease materials. Examples of these pairs are: (1) purified epithelial cell populations derived from human breast tumours, matched to purified normal populations of human breast epithelial cells, and (2) the invading pathogenic hyphal form of *C. albicans*, matched to the non-invading yeast form of *C. albicans*. When the proteome images from each pair are aligned, the Proteograph™ software is able to rapidly identify those proteins (each referenced as having a unique molecular cluster index, or MCI) that are either unique, or those that are differentially expressed. Thus, the Proteograph output from this analysis is both qualitative and quantitative.

Proteograph analysis for a particular study can also be undertaken on any number of samples. For example, one might compare anything from a few to several hundred preparations or samples, each from a normal and disease counterpart, and have these analysed in a single Proteograph study. In this way, it is possible to assign strong statistical confidence to the data and in some instances to identify specific subpopulations within the input biological sources. This feature will become increasingly significant in the near future, and there is a clear synergy here whereby proteomics can work closely with pharmacogenomic approaches to stratify patient populations and achieve effective targeted care for the patient. Whatever the source of the materials, the net output of Proteograph analysis is immediate identification of disease specific proteins. This is shown in Fig. 3, which shows the results of a proteograph obtained by comparing untreated human hepatoma cells with cells following exposure to a clinical

Figure 2. *Representative proteomes obtained from (a) human serum, (b) the pathogenic fungus Candida albicans and (c) the human hepatoma cell line Huh7.*

mass spectrometry facility coupled to advanced databases to annotate these MCIs as individual proteins is applied. As these are all disease specific proteins, each could represent a novel target and/or a novel disease marker. The process becomes even more powerful when a panel of features, rather than individual features, are assigned. The relevance of this is apparent when one considers that most diseases, if not all, are multifactorial in nature and arise from polygenic changes. Rather than analysing events in isolation, the ability to examine hundreds or thousands of events simultaneously, as shown by proteomics, can offer real advantages.

Identification and assignment of candidate targets

The rapid identification and assignment of candidate targets and markers represents a huge challenge, but this has been greatly facilitated by combining the recent advances made in proteomics and analytical mass spectrometry[9]. Using automated procedures it is now possible to annotate proteins present in femtomole quantities, which would depict the low abundance class of proteins. The process of annotation is similarly aided by the quality and richness of the sequence specific databases that are currently available, both in the public domain and in the private sector (e.g. those supplied by Incyte Pharmaceuticals). In this respect, the advances in proteomics have benefited considerably from the breakthroughs achieved with genomics.

From an application perspective, cancer studies provide a good opportunity whereby proteomics can be instrumental in identifying disease specific proteins, because it is often feasible to obtain normal and diseased tissue from the same patient. For example, proteomic studies have been reported on neuroblastomas[10], human breast proteins from normal and tumour sources[11–13], lung tumours[14], colon tumours[15] and bladder tumours[16]. There are also proteomic studies reported within the cardiovascular therapeutic area, in which disease or response proteins are identified[17,18].

Genomic microarray analysis can similarly identify unique species or clusters of mRNAs that are disease specific. However, in some instances, there is a clear lack of correlation between the levels of a specific mRNA and its corresponding protein (Ref. 19, Gypi, S.P. et al., submitted). This has now been noted by many investigators and reaffirms that post-transcriptional events, including protein stability, protein modification (such as phosphorylation, glycosylation, acylation and methylation) and cell localization, can constitute major regulatory steps. Proteomic analysis captures all of these steps and can therefore provide unique and valuable information independent from, or complementary to, genomic data.

Rosetta

Foregrounds:　Huh7 cells treated with 5FU
Backgrounds:　Huh7 cells untreated
■■■■■■■■　Upregulated in Huh7 cells treated with 5FU with respect to untreated Huh7 cells
■■■■■■■■　Downregulated in Huh7 cells treated with 5FU with respect to untreated Huh7 cells

MCI	Fold change		pI	MW
3589	16.3		5.60	13718
4035	15.8		8.73	13224
4033	12.3		8.94	13117
4052	11.7		8.96	21434
4064	11.1		9.37	29389
3792	10.9		8.75	41162
3976	10.5		7.14	12061
3672	10.5		8.83	24121
3986	10.4		4.61	10853
3759	9.9		4.98	41420
4032	9.7		4.56	13117
1972	−9.4	■■■■■	7.92	12396
3587	9.4		4.50	14954
2033	−9.4	■■■■■	7.93	11126
3984	8.9		5.32	11090
2403	−8.8	■■■■■	6.04	25950
3748	8.8		6.32	35513
2105	−8.7	■■■■■	4.76	20803
3897	8.7		4.94	87842
4221	8.6		5.78	71963

Figure 3. Table of differential protein expression profiles, referred to as a Rosetta Proteograph™, between Huh7 cells with and without the cytotoxic agent 5-FU. Bars are quantized and do not represent exact fold change values.

cytotoxic agent. In this instance, only the top 20 differentially expressed MCIs are shown, but the readout would normally extend to a defined cut-off value, typically a two-fold or greater difference in expression levels, determined by the user.

In a typical analysis involving disease and normal mammalian material, in which each proteome would have ~2000 protein features each assigned an MCI, the proteograph might identify somewhere in the region of 50–300 MCIs that are unique or differentially expressed. To capitalize rapidly on these data, at OGS a high-throughput

Proteomics for target validation and signal transduction studies

The identification of disease specific proteins alone is insufficient to begin a drug screening process. It is critical to assign function and validation to these proteins by confirming they are indeed pivotal in the disease process. These studies need to encompass both gain- and loss-of-function analyses. This would determine whether the activity of a candidate target (an enzyme, for example), eliminated by molecular/cellular techniques, could reverse a disease phenotype. If this happened, then the investigator would have increased confidence that a small-molecule inhibitor against the target would also have a similar effect. The proposal of candidate drug targets is often not a difficult process, but validating them is another matter. Validation represents a major bottleneck where the wrong decision can have serious consequences[20].

Proteomics can be used to evaluate the role of a chosen target protein in signal transduction cascades directly relevant to the disease. In this manner, valuable information is forthcoming on the signalling pathways that are perturbed by a target protein and how they might be corrected by appropriate therapeutics. Techniques that are well established in one-dimensional protein studies to investigate signalling pathways, such as western blotting and immunoprecipitation, are highly suited to proteomic applications. For example, the proteomes obtained can be blotted onto membranes and probed with antibodies against the target protein or related signalling molecules[21-23]. Because proteomics can resolve >2000 proteins on a single gel, it is possible to derive important information on specific isoforms (such as glycosylated or phosphorylated variants) of signalling molecules. This will result in characterization of how they are altered in the disease process. Western immunoblotting techniques using high-affinity antibodies will typically identify proteins present at ~10 copies per cell (~1.7 fmol); this is in contrast to the best fluorescent dyes currently available that are limited to imaging proteins at 1000 or more copies per cell. The level of sensitivity derived by these applications will greatly facilitate interpretation of complex signalling pathways and contribute significantly to validation of the target under study.

Immunoprecipitation studies

Similarly, immunoprecipitation studies are another useful way to exploit the resolving power of proteomics[24,25]. In this instance, very large quantities of protein (e.g. several milligrams) can be subjected to incubation with antibodies against chosen signalling molecules. This allows high-affinity capture of these proteins, which can subsequently be eluted and electrophoresed on a 2D gel to provide a high-resolution proteome of a specific subset of proteins. Detection by blot analysis allows the identification of extremely small amounts of defined signalling molecules. Again, the different isoforms of even very low abundance proteins can be seen, and, very importantly, the technique allows the investigator to identify multiprotein complexes or other proteins that co-precipitate with the target protein. These coassociating proteins frequently represent signalling partners for the target protein, and their identification by mass spectrometry can lead to invaluable information on the signalling processes involved.

The depth of signal transduction analysis offered by proteomics, and the utility for target validation studies, can be extended even further by applying cell fractionation studies[26-28]. By purifying subcellular fractions, such as membrane, nuclear, organelle and cytosolic, it is possible to assign a localization to proteins of interest and to follow their trafficking in a cell. Enrichment of these fractions will also allow much higher representation of low abundance proteins on the proteome. Their detection by fluorescent dyes or immunoblot techniques will lead to the identification of proteins in the range of 1–10 copies per cell, putting the sensitivity on a par with genomic approaches.

These signal transduction analyses can be of additional value in experiments where inhibitors derived from a screening programme against the target are being evaluated for their potency and selectivity. The inhibitors can encompass small molecules, antisense nucleic acid constructs, dominant-negative proteins, or neutralizing antibodies microinjected into cells. In each case, proteome analysis can provide unique data in support of validation studies for a chosen candidate drug target.

Proteomics and drug mode-of-action studies

Once a validated target is committed to a screening regimen to identify and advance a lead molecule, it is important to confirm that the efficacy of the inhibitor is through the expected mechanism. Such mode-of-action studies are usually tackled by various cell biological and biochemical methods. Proteomics can also be usefully applied to these studies and this is illustrated below by describing data obtained with OGT719. This is a novel galactosyl derivative of the cytotoxic agent 5-fluorouracil (5-FU), which is currently being developed by OGS for the treatment of hepatocellular carcinoma and colorectal metastases localized in the liver. The premise underpinning the design and rationale of OGT719 was to derive a 5-FU prodrug capable

Figure 4. Features that are specifically up- or downregulated in Huh7 cells by either 5-fluorouracil (5-FU) or OGT719: (a) elongation factor 1α2, (b) novel (three peptides by MS-MS) and (c) α-subunit of prolyl-4-hydroxylase. Arrows indicate up- or downregulated.
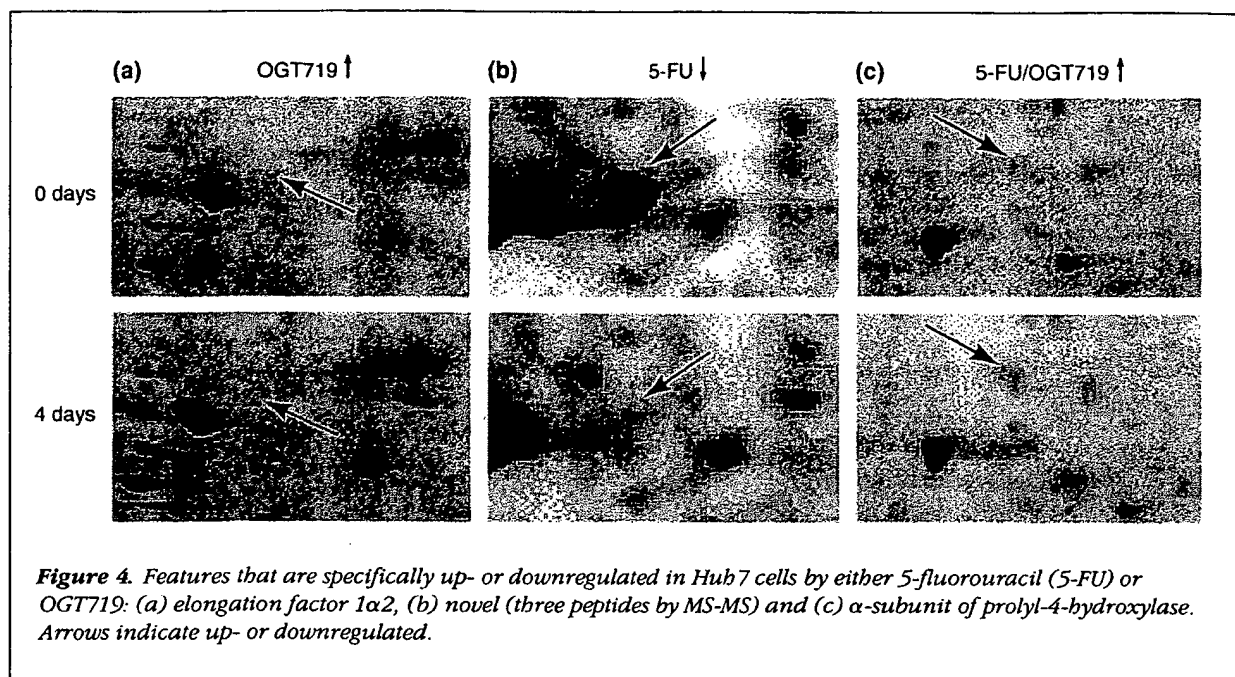
of targeting, and being retained in, cells bearing the asialo-glycoprotein receptor (ASGP-r), including hepatocytes[29], hepatoma Huh7 cells[30] and some colorectal tumour cells[31]. The growth of the human hepatoma cell line Huh7 is inhibited by 5-FU or by OGT719. If the inhibition by OGT719 were the result of uptake and conversion to 5-FU as the active component, then it would be expected that Huh7 cells would show similar proteome profiles following exposure to either drug.

To examine these possibilities, we conducted an experiment taking samples of Huh7 cells that had been treated with IC_{50} doses of either OGT719 or 5-FU. Total cell lysates were prepared and taken through 2D electrophoresis, fluorescence staining, digital imaging and Proteograph analysis. To facilitate the interpretation of the data across all of the 2291 features seen on the proteomes, drug-induced protein changes of fivefold or greater, identified by the Proteograph, were analysed further. Interestingly, from this analysis 19 identical proteins were changed fivefold or more by both drugs, strongly suggesting similarities in the mode of action for these two compounds.

Thus, from very complex data involving >2000 protein features, using proteomics it is possible to analyse quantitatively and qualitatively each protein during its exposure to drugs. The biologist is now able to focus a series of further studies specifically on an enriched subset of proteins.

Figure 4 shows highlighted examples of the selected areas of the proteome where some of these identified proteins in the above study are altered in response to either or both drugs.

Several of the proteins identified above as being modulated similarly by 5-FU or OGT719 in Huh7 cells were subjected to tandem mass-spectrometric analysis for annotation. Some of these, such as the nuclear ribosomal RNA-binding protein[32], can be placed into pyrimidine pathways or related cell cycle/growth biochemical pathways in which 5-FU is known to act.

To attribute further significance to the proteome mode-of-action studies with OGT719, another cell line, the rat sarcoma HSN, was used. Growth of these cells is inhibited by 5-FU, but they are completely refractory to OGT719; notably they lack the ASGP-r, which might explain this finding (unpublished). For our proteome studies, HSN cells were treated with 5-FU or OGT719 over a time course of one, two and four days. At each time point, cells were harvested and processed to derive proteomes and Proteographs. As before, we purposely focused on those proteins that increased or decreased by fivefold or more. In this instance, there were no proteins co-modulated by the two drugs. This is perhaps to be expected, given that the HSN cells are killed by 5-FU and yet are refractory to OGT719.

Clear potential

The above is just an example of how proteomics can be used to address the mode of action of anticancer drugs. The potential of this approach is clear, and one can envisage situations where it will be profitable to compare the proteomes of cells in which the drug target has been eliminated by molecular knockout techniques, or with small-molecule inhibitors believed to act specifically on the same target. In addition to using proteomics to examine the action of drugs, it is also possible to use this approach to gauge the extent of nonspecific effects that might eventually lead to toxicity. For instance, in the example used above with HSN cells treated with OGT719, although cell growth was not affected, the levels of several specific proteins were changed. Further investigation of these proteins and the signalling pathways in which they are involved could be illuminating in predicting the likelihood or otherwise of long-term toxicity.

Use of proteomics in formal drug toxicology studies

A drug discovery programme at the stage where leads have been identified and mode-of-action studies are advanced, will proceed to investigate the pharmacokinetic and toxicology profile of those agents. These two parameters are of major importance in the drug discovery process, and many agents that have looked highly promising from *in vitro* studies have subsequently failed because of insurmountable pharmacokinetic and/or toxicity problems *in vivo*. Whereas the pharmacokinetic properties of a molecule can now be characterized quickly and accurately, toxicity studies are typically much longer and more demanding in their interpretation.

The ability to achieve fast and accurate predictions of toxicity within an *in vivo* setting would represent a big step forward in accelerating any drug discovery programme. Toxicity from a drug can be manifested in any organ. However, because the liver and kidney are the major sites in the body responsible for metabolism and elimination of most drugs, it is informative to examine these particular organs in detail to provide early indications about events that might result in toxicity.

The basis for most xenobiotic metabolizing activity is to increase the hydrophilicity of the compound and so facilitate its removal from the body. Most drugs are metabolized in the liver via the cytochrome P450 family of enzymes, which are known to comprise a total of ~200 different members[33,34], encompassing a wide array of overlapping specificities for different substrates. In addition to clearance, they also play a major role in metabo-

lism that can lead to the production and removal of toxic species, and in some instances it is possible to correlate the ability or failure to remove such a toxin with a specific P450 or subgroup.

Unique P450 profiles

Each individual person will have a slightly different P450 profile, largely from polymorphisms and changes in expression levels, although other genetic and environmental factors aside from P450 also need to be taken into consideration. A significant amount of research is currently being directed towards this field – known as pharmacogenomics – with the aim of predicting how a patient will respond to a drug, as determined by their genetic make-up[35-37]. The marked variation of individuals in their ability to clear a compound can be one of the key factors in deciding the overall pharmacokinetic profile of a drug. Not only will this have a bearing on the likelihood of a patient responding to a treatment, but it will also be a factor in determining the possibility of their experiencing an adverse effect.

Many pharmaceutical companies are already employing genomic approaches, involving P450 measurements, as a key step in their assessment of the toxicological profile of a candidate drug and therefore of its suitability, or otherwise, to be considered for human clinical trials. There are limits to this approach, however. Whereas the P450 mRNA profiling can predict with some accuracy the likely metabolic fate of a drug, it will not provide information on whether the metabolites would subsequently lead to toxicity. Besides the patient-to-patient differences in steady-state levels of the P450s, there are also characteristic induction responses of these enzymes to some drugs. Moreover, as there can be some doubt over the correlation of mRNA levels and the corresponding protein levels, there is scope for misinterpretation of the results and hence real advantages to be gained from a proteome approach. In both instances, the ability to examine entire proteome profiles, including the P450 proteins, will be a significant advantage in understanding and predicting the metabolism and toxicological outcome of drugs.

In addition to direct organ and tissue studies, the serum, which collects the majority of toxicity markers released from susceptible organs and tissues throughout the entire body, can be utilized. Serum is rich in nuclease activity and, as pharmacogenomics is not suited to deal with these samples, valuable markers of toxicity could go undetected. However, by using proteomics for these types of analyses, serum markers (and clusters thereof) are now accessible for evaluation as indicators of toxicity.

Pharmacoproteomics

Proteomics can thus be used to add a new sphere of analysis to the study of toxicity at the protein level, and in the era of '-omics' there is a case to be made to adopt the term 'Pharmacoproteomics™'. Animals can be dosed with increasing levels of an experimental drug over time, and serum samples can be drawn for consecutive proteome analyses. Using this procedure, it should be possible to identify individual markers, or clusters thereof, that are dose related and correlate with the emergence and severity of toxicity. Markers might appear in the serum at a defined drug dose and time that are predictive of early toxicity within certain organs and if allowed to continue will have damaging consequences. These serum markers could subsequently be used to predict the response of each individual and allow tailoring of therapy whereby optimal efficacy is achieved without adverse side effects being apparent. This application can obviously extend to tracking toxicity of drugs in clinical trials where serum can be readily drawn and analysed. Surrogate markers for drug efficacy could also be detected by this procedure and could facilitate the challenge of identifying patient classes who will respond favourably to a drug and at what dosage.

Conclusions

By contrast to the agents administered to patients in clinical wards, the process of drug discovery is not a prescriptive series of steps. The risks are high and there are long timelines to be endured before it is known whether a candidate drug will succeed or fail. At each step of the drug discovery process there is often scope for flexibility in interpretation, which over many steps is cumulative. The pharmaceutical companies most likely to succeed in this environment are those that are able to make informed accurate decisions within an accelerated process.

The genomics revolution has impacted very positively upon these issues and now has a powerful new partner in proteomics. The ability to undertake global analysis of proteins from a very wide diversity of biological systems and to interrogate these in a high-throughput, systematic manner will add a significant new dimension to drug discovery. Each step of the process from target discovery to clinical trials is accessible to proteomics, often providing unique sets of data. Using the combination of genomics and proteomics, scientists can now see every dimension of their biological focus, from genes, mRNA, proteins and their subcellular localization. This will greatly assist our understanding of the fundamental mechanistic basis of human disease and allow new improved and speedier drug discovery strategies to be implemented.

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U01 AA13518-01 Adron Harris (PI) 25% effort
9/28/01 - 9/27/06
NIH/NIAAA
"INIA: Microarray Core"
This proposal was a response to the Integrative Neuroscience Initiative on Alcoholism (INIA) RFA-AA-01-002. The overall goal is to support the use of microarray technology to define changes in gene expression that either predict or accompany excessive alcohol consumption.
Role: Co-investigator

003658-0223-2001 Iyer (PI) 16% effort
01/01/02 - 08/31/04
Texas Higher Education Coordinating Board (ARP)
"Microarray based global mapping of DNA-protein interactions at promoters in human cells"
This is a pilot project to map the in vivo interactions of transcription factors with human promoters
Role: PI

Information Technology Research 0325116 R. Mooney (PI) 9% effort
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NSF
"Feedback from Multi-Source Data Mining to Experimentation for Gene Network Discovery"
Role: Co-investigator

1 R01 CA95548-01A2 (pending) Iyer (PI) 25% effort
12/1/03 – 11/30/08
NIH
"Analysis of genome-wide transcriptional control in yeast"
This is a project to identify stress responsive transcription factor targets in yeast through the use of DNA microarrays
Role: PI

Breast Cancer Idea Award (pending) Iyer (PI) 10% effort
1/1/04 – 12/31/06
US Army Medical Research and Materiel Command
"Genome-wide chromosomal targets of oncogenic transcription factors"
This is a project aimed at identifying direct chromosomal targets of c-myc and ER in human cells through the use of a novel sequence tag analysis method.
Role: PI
003658-0531-2003 (pending) Marcotte (PI) 8% effort
01/01/04 - 12/31/05
Texas Higher Education Coordinating Board (ATP)
"Cell arrays: A novel high–throughput platform for measuring gene function on a genomic scale"
This proposal is aimed at developing a novel microarray based platform for automated, high–throughput microscopic imaging of cells, allowing rapid and systematic evaluation of gene function.

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Exploring the Metabolic and Genetic Control of Gene Expression on a Genomic Scale

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DNA microarrays containing virtually every gene of *Saccharomyces cerevisiae* were used to carry out a comprehensive investigation of the temporal program of gene expression accompanying the metabolic shift from fermentation to respiration. The expression profiles observed for genes with known metabolic functions pointed to features of the metabolic reprogramming that occur during the diauxic shift, and the expression patterns of many previously uncharacterized genes provided clues to their possible functions. The same DNA microarrays were also used to identify genes whose expression was affected by deletion of the transcriptional co-repressor *TUP1* or overexpression of the transcriptional activator *YAP1*. These results demonstrate the feasibility and utility of this approach to genomewide exploration of gene expression patterns.

The complete sequences of nearly a dozen microbial genomes are known, and in the next several years we expect to know the complete genome sequences of several metazoans, including the human genome. Defining the role of each gene in these genomes will be a formidable task, and understanding how the genome functions as a whole in the complex natural history of a living organism presents an even greater challenge.

Knowing when and where a gene is expressed often provides a strong clue as to its biological role. Conversely, the pattern of genes expressed in a cell can provide detailed information about its state. Although regulation of protein abundance in a cell is by no means accomplished solely by regulation of mRNA, virtually all differences in cell type or state are correlated with changes in the mRNA levels of many genes. This is fortuitous because the only specific reagent required to measure the abundance of the mRNA for a specific gene is a cDNA sequence. DNA microarrays, consisting of thousands of individual gene sequences printed in a high-density array on a glass microscope slide (*1*, *2*), provide a practical and economical tool for studying gene expression on a very large scale (3–6).

Saccharomyces cerevisiae is an especially

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favorable organism in which to conduct a systematic investigation of gene expression. The genes are easy to recognize in the genome sequence, *cis* regulatory elements are generally compact and close to the transcription units, much is already known about its genetic regulatory mechanisms, and a powerful set of tools is available for its analysis.

A recurring cycle in the natural history of yeast involves a shift from anaerobic (fermentation) to aerobic (respiration) metabolism. Inoculation of yeast into a medium rich in sugar is followed by rapid growth fueled by fermentation, with the production of ethanol. When the fermentable sugar is exhausted, the yeast cells turn to ethanol as a carbon source for aerobic growth. This switch from anaerobic growth to aerobic respiration upon depletion of glucose, referred to as the diauxic shift, is correlated with widespread changes in the expression of genes involved in fundamental cellular processes such as carbon metabolism, protein synthesis, and carbohydrate storage (*7*). We used DNA microarrays to characterize the changes in gene expression that take place during this process for nearly the entire genome, and to investigate the genetic circuitry that regulates and executes this program.

Yeast open reading frames (ORFs) were amplified by the polymerase chain reaction (PCR), with a commercially available set of primer pairs (*8*). DNA microarrays, containing approximately 6400 distinct DNA sequences, were printed onto glass slides by

using a simple robotic printing device (*9*). Cells from an exponentially growing culture of yeast were inoculated into fresh medium and grown at 30°C for 21 hours. After an initial 9 hours of growth, samples were harvested at seven successive 2-hour intervals, and mRNA was isolated (*10*). Fluorescently labeled cDNA was prepared by reverse transcription in the presence of Cy3(green)- or Cy5(red)-labeled deoxyuridine triphosphate (dUTP) (*11*) and then hybridized to the microarrays (*12*). To maximize the reliability with which changes in expression levels could be discerned, we labeled cDNA prepared from cells at each successive time point with Cy5, then mixed it with a Cy3-labeled "reference" cDNA sample prepared from cells harvested at the first interval after inoculation. In this experimental design, the relative fluorescence intensity measured for the Cy3 and Cy5 fluors at each array element provides a reliable measure of the relative abundance of the corresponding mRNA in the two cell populations (Fig. 1). Data from the series of seven samples (Fig. 2), consisting of more than 43,000 expression-ratio measurements, were organized into a database to facilitate efficient exploration and analysis of the results. This database is publicly available on the Internet (*13*).

During exponential growth in glucose-rich medium, the global pattern of gene expression was remarkably stable. Indeed, when gene expression patterns between the first two cell samples (harvested at a 2-hour interval) were compared, mRNA levels differed by a factor of 2 or more for only 19 genes (0.3%), and the largest of these differences was only 2.7-fold (*14*). However, as glucose was progressively depleted from the growth media during the course of the experiment, a marked change was seen in the global pattern of gene expression. mRNA levels for approximately 710 genes were induced by a factor of at least 2, and the mRNA levels for approximately 1030 genes declined by a factor of at least 2. Messenger RNA levels for 183 genes increased by a factor of at least 4, and mRNA levels for 203 genes diminished by a factor of at least 4. About half of these differentially expressed genes have no currently recognized function and are not yet named. Indeed, more than 400 of the differentially expressed genes have no apparent homology

to any gene whose function is known (15). The responses of these previously uncharacterized genes to the diauxic shift therefore provides the first small clue to their possible roles.

The global view of changes in expression of genes with known functions provides a vivid picture of the way in which the cell adapts to a changing environment. Figure 3 shows a portion of the yeast metabolic pathways involved in carbon and energy metabolism. Mapping the changes we observed in the mRNAs encoding each enzyme onto this framework allowed us to infer the redirection in the flow of metabolites through this system. We observed large inductions of the genes coding for the enzymes aldehyde dehydrogenase (ALD2) and acetyl–coenzyme A(CoA) synthase (ACS1), which function together to convert the products of alcohol dehydrogenase into acetyl-CoA, which in turn is used to fuel the tricarboxylic acid (TCA) cycle and the glyoxylate cycle. The concomitant shutdown of transcription of the genes encoding pyruvate decarboxylase and induction of pyruvate carboxylase rechannels pyruvate away from acetaldehyde, and instead to oxalacetate, where it can serve to supply the TCA cycle and gluconeogenesis. Induction of the pivotal genes PCK1, encoding phosphoenolpyruvate carboxykinase, and FBP1, encoding fructose 1,6-biphosphatase, switches the directions of two key irreversible steps in glycolysis, reversing the flow of metabolites along the reversible steps of the glycolytic pathway toward the essential biosynthetic precursor, glucose-6-phosphate. Induction of the genes coding for the trehalose synthase and glycogen synthase complexes promotes channeling of glucose-6-phosphate into these carbohydrate storage pathways.

Just as the changes in expression of genes encoding pivotal enzymes can provide insight into metabolic reprogramming, the behavior of large groups of functionally related genes can provide a broad view of the systematic way in which the yeast cell adapts to a changing environment (Fig. 4). Several classes of genes, such as cytochrome c–related genes and those involved in the TCA/glyoxylate cycle and carbohydrate storage, were coordinately induced by glucose exhaustion. In contrast, genes devoted to protein synthesis, including ribosomal proteins, tRNA synthetases, and translation, elongation, and initiation factors, exhibited a coordinated decrease in expression. More than 95% of ribosomal genes showed at least twofold decreases in expression during the diauxic shift (Fig. 4) (13). A noteworthy and illuminating exception was that the

genes encoding mitochondrial ribosomal genes were generally induced rather than repressed after glucose limitation, highlighting the requirement for mitchondrial biogenesis (13). As more is learned about the functions of every gene in the yeast genome, the ability to gain insight into a cell's response to a changing environment through its global gene expression patterns will become increasingly powerful.

Several distinct temporal patterns of expression could be recognized, and sets of genes could be grouped on the basis of the similarities in their expression patterns. The characterized members of each of these groups also shared important similarities in their functions. Moreover, in most cases, common regulatory mechanisms could be inferred for sets of genes with similar expression profiles. For example, seven genes showed a late induction profile, with mRNA levels increasing by more than ninefold at

the last timepoint but less than threefold at the preceding timepoint (Fig. 5B). All of these genes were known to be glucose-repressed, and five of the seven were previously noted to share a common upstream activating sequence (UAS), the carbon source response element (CSRE) (16–20). A search in the promoter regions of the remaining two genes, ACR1 and IDP2, revealed that ACR1, a gene essential for ACS1 activity, also possessed a consensus CSRE motif, but interestingly, IDP2 did not. A search of the entire yeast genome sequence for the consensus CSRE motif revealed only four additional candidate genes, none of which showed a similar induction.

Examples from additional groups of genes that shared expression profiles are illustrated in Fig. 5, C through F. The sequences upstream of the named genes in Fig. 5C all contain stress response elements (STRE), and with the exception

Fig. 1. Yeast genome microarray. The actual size of the microarray is 18 mm by 18 mm. The microarray was printed as described (9). This image was obtained with the same fluorescent scanning confocal microscope used to collect all the data we report (49). A fluorescently labeled cDNA probe was prepared from mRNA isolated from cells harvested shortly after inoculation (culture density of <5 × 10^6 cells/ml and media glucose level of 19 g/liter) by reverse transcription in the presence of Cy3-dUTP. Similarly, a second probe was prepared from mRNA isolated from cells taken from the same culture 9.5 hours later (culture density of ~2 × 10^8 cells/ml, with a glucose level of <0.2 g/liter) by reverse transcription in the presence of Cy5-dUTP. In this image, hybridization of the Cy3-dUTP–labeled cDNA (that is, mRNA expression at the initial timepoint) is represented as a green signal, and hybridization of Cy5-dUTP–labeled cDNA (that is, mRNA expression at 9.5 hours) is represented as a red signal. Thus, genes induced or repressed after the diauxic shift appear in this image as red and green spots, respectively. Genes expressed at roughly equal levels before and after the diauxic shift appear in this image as yellow spots.

of HSP42, have previously been shown to be controlled at least in part by these elements (21–24). Inspection of the sequences upstream of HSP42 and the two uncharacterized genes shown in Fig. 5C, YKL026c, a hypothetical protein with similarity to glutathione peroxidase, and YGR043c, a putative transaldolase, revealed that each of these genes also possess repeated upstream copies of the stress-responsive CCCCT motif. Of the 13 additional genes in the yeast genome that shared this expression profile [including HSP30, ALD2, OM45, and 10 uncharacterized ORFs (25)], nine contained one or more recognizable STRE sites in their upstream regions.

The heterotrimeric transcriptional activator complex HAP2,3,4 has been shown to be responsible for induction of several genes important for respiration (26–28). This complex binds a degenerate consensus sequence known as the CCAAT box (26). Computer analysis, using the consensus sequence TNRYTGGB (29), has suggested that a large number of genes involved in respiration may be specific targets of HAP2,3,4 (30). Indeed, a putative HAP2,3,4 binding site could be found in the sequences upstream of each of the seven cytochrome c–related genes that showed the greatest magnitude of induction (Fig. 5D). Of 12 additional cytochrome c–related genes that were induced, HAP2,3,4 binding sites were present in all but one. Significantly, we found that transcription of HAP4 itself was induced nearly ninefold concomitant with the diauxic shift.

Control of ribosomal protein biogenesis is mainly exerted at the transcriptional level, through the presence of a common upstream-activating element (UAS_{rpg}) that is recognized by the Rap1 DNA-binding protein (31, 32). The expression profiles of seven ribosomal proteins are shown in Fig. 5F. A search of the sequences upstream of all seven genes revealed consensus Rap1-binding motifs (33). It has been suggested that declining Rap1 levels in the cell during starvation may be responsible for the decline in ribosomal protein gene expression (34). Indeed, we observed that the abundance of RAP1 mRNA diminished by 4.4-fold, at about the time of glucose exhaustion.

Of the 149 genes that encode known or putative transcription factors, only two, HAP4 and SIP4, were induced by a factor of more than threefold at the diauxic shift. SIP4 encodes a DNA-binding transcriptional activator that has been shown to interact with Snf1, the "master regulator" of glucose repression (35). The eightfold induction of SIP4 upon depletion of glucose strongly suggests a role in the induction of

downstream genes at the diauxic shift.

Although most of the transcriptional responses that we observed were not previously known, the responses of many genes during the diauxic shift have been described. Comparison of the results we obtained by DNA microarray hybridization with previously reported results therefore provided a strong test of the sensitivity and accuracy of this approach. The expression patterns we observed for previously characterized genes showed almost perfect concordance with previously published results (36). Moreover, the differential expression measurements obtained by DNA microarray hybridization were reproducible in duplicate experiments. For example, the remarkable changes in gene expression between cells harvested immediately after inoculation and immediately after the diauxic shift (the first and sixth intervals in this time series) were measured in duplicate, independent DNA microarray hybridizations. The correlation coefficient for two complete sets of expression ratio measurements was 0.87, and for more than 95% of the genes, the expres-

sion ratios measured in these duplicate experiments differed by less than a factor of 2. However, in a few cases, there were discrepancies between our results and previous results, pointing to technical limitations that will need to be addressed as DNA microarray technology advances (37, 38). Despite the noted exceptions, the high concordance between the results we obtained in these experiments and those of previous studies provides confidence in the reliability and thoroughness of the survey.

The changes in gene expression during this diauxic shift are complex and involve integration of many kinds of information about the nutritional and metabolic state of the cell. The large number of genes whose expression is altered and the diversity of temporal expression profiles observed in this experiment highlight the challenge of understanding the underlying regulatory mechanisms. One approach to defining the contributions of individual regulatory genes to a complex program of this kind is to use DNA microarrays to identify genes whose expression is affected

Fig. 2. The section of the array indicated by the gray box in Fig. 1 is shown for each of the experiments described here. Representative genes are labeled. In each of the arrays used to analyze gene expression during the diauxic shift, red spots represent genes that were induced relative to the initial timepoint, and green spots represent genes that were repressed relative to the initial timepoint. In the arrays used to analyze the effects of the tup1Δ mutation and YAP1 overexpression, red spots represent genes whose expression was increased, and green spots represent genes whose expression was decreased by the genetic modification. Note that distinct sets of genes are induced and repressed in the different experiments. The complete images of each of these arrays can be viewed on the Internet (13). Cell density as measured by optical density (OD) at 600 nm was used to measure the growth of the culture.

Growth OD 0.14

Growth OD 0.46

Growth OD 0.8
Putative homocitrate synthase

Growth OD 1.8
ACO1

Growth OD 3.7
GLK1
TEF4

Growth OD 6.9
RPL15A
YHR037
PEM2

Growth OD 7.3
CIT2
STL1
YHR085

Δ tup1
YCR104 Flo1
homolog
Homology to maltase
Tip1 homolog

YAP1 +++
Aryl-alcohol oxidoreductase
Bacterial CsgA homolog
YAP1

by mutations in each putative regulatory gene. As a test of this strategy, we analyzed the genomewide changes in gene expression that result from deletion of the *TUP1* gene. Transcriptional repression of many genes by glucose requires the DNA-binding repressor Mig1 and is mediated by recruiting the transcriptional co-repressors Tup1 and Cyc8/Ssn6 (39). Tup1 has also been implicated in repression of oxygen-regulated, mating-type-specific, and DNA-damage-inducible genes (40).

Wild-type yeast cells and cells bearing a deletion of the *TUP1* gene (*tup1*Δ) were grown in parallel cultures in rich medium containing glucose as the carbon source. Messenger RNA was isolated from exponentially growing cells from the two populations and used to prepare cDNA labeled with Cy3 (green) and Cy5 (red), respectively (11). The labeled probes were mixed and simultaneously hybridized to the microarray. Red spots on the microarray therefore represented genes whose transcription was induced in the *tup1*Δ strain, and thus presumably repressed by Tup1 (41). A representative section of the microarray (Fig. 2, bottom middle panel) illustrates that the genes whose expression was affected by the *tup1*Δ mutation, were, in general, distinct from those induced upon glucose exhaustion [complete images of all the arrays shown in Fig. 2 are available on the Internet (13)]. Nevertheless, 34 (10%) of the genes that were induced by a factor of at least 2 after the diauxic shift were similarly induced by deletion of *TUP1*, suggesting that these genes may be subject to *TUP1*-mediated repression by glucose. For example, *SUC2*, the gene encoding invertase, and all five hexose transporter genes that were induced during the course of the diauxic shift were similarly induced, in duplicate experiments, by the deletion of *TUP1*.

The set of genes affected by Tup1 in this experiment also included α-glucosidases, the mating-type-specific genes *MFA1* and *MFA2*, and the DNA damage-inducible *RNR2* and *RNR4*, as well as genes involved in flocculation and many genes of unknown function. The hybridization signal corresponding to expression of *TUP1* itself was also severely reduced because of the (incomplete) deletion of the transcription unit in the *tup1*Δ strain, providing a positive control in the experiment (42).

Many of the transcriptional targets of Tup1 fell into sets of genes with related biochemical functions. For instance, although only about 3% of all yeast genes appeared to be *TUP1*-repressed by a factor of more than 2 in duplicate experiments under these conditions, 6 of the 13 genes that have been implicated in flocculation (15) showed a reproducible increase in expression of at least twofold when *TUP1* was deleted. Another group of related genes that appeared to be subject to *TUP1* repression encodes the serine-rich cell wall mannoproteins, such as Tip1 and Tir1/Srp1 which are induced by cold shock and other stresses (43), and similar, serine-poor proteins, the seripauperins (44). Messenger RNA levels for 23 of the 26 genes in this group were reproducibly elevated by at least 2.5-fold in the *tup1*Δ

Fig. 3. Metabolic reprogramming inferred from global analysis of changes in gene expression. Only key metabolic intermediates are identified. The yeast genes encoding the enzymes that catalyze each step in this metabolic circuit are identified by name in the boxes. The genes encoding succinyl-CoA synthase and glycogen-debranching enzyme have not been explicitly identified, but the ORFs YGR244 and YPR184 show significant homology to known succinyl-CoA synthase and glycogen-debranching enzymes, respectively, and are therefore included in the corresponding steps in this figure. Red boxes with white lettering identify genes whose expression increases in the diauxic shift. Green boxes with dark green lettering identify genes whose expression diminishes in the diauxic shift. The magnitude of induction or repression is indicated for these genes. For multimeric enzyme complexes, such as succinate dehydrogenase, the indicated fold-induction represents an unweighted average of all the genes listed in the box. Black and white boxes indicate no significant differential expression (less than twofold). The direction of the arrows connecting reversible enzymatic steps indicate the direction of the flow of metabolic intermediates, inferred from the gene expression pattern, after the diauxic shift. Arrows representing steps catalyzed by genes whose expression was strongly induced are highlighted in red. The broad gray arrows represent major increases in the flow of metabolites after the diauxic shift, inferred from the indicated changes in gene expression.

strain, and 18 of these genes were induced by more than sevenfold when *TUP1* was deleted. In contrast, none of 83 genes that could be classified as putative regulators of the cell division cycle were induced more than twofold by deletion of *TUP1*. Thus, despite the diversity of the regulatory systems that employ Tup1, most of the genes that it regulates under these conditions fall into a limited number of distinct functional classes.

Because the microarray allows us to monitor expression of nearly every gene in yeast, we can, in principle, use this approach to identify all the transcriptional targets of a regulatory protein like Tup1. It is important to note, however, that in any single experiment of this kind we can only recognize those target genes that are normally repressed (or induced) under the conditions of the experiment. For instance, the experiment described here analyzed a *MAT* α strain in which *MFA1* and *MFA2*, the genes encoding the a-factor mating pheromone precursor, are normally repressed. In the isogenic *tup1*Δ strain, these genes were inappropriately expressed, reflecting the role that Tup1 plays in their repression. Had we instead carried out this experiment with a *MAT*A strain (in which expression of *MFA1* and *MFA2* is not repressed), it would not have been possible to conclude anything regarding the role of Tup1 in the repression of these genes. Conversely, we cannot distinguish indirect effects of the chronic absence of Tup1 in the mutant strain from effects directly attributable to its participation in repressing the transcription of a gene.

Another simple route to modulating the activity of a regulatory factor is to overexpress the gene that encodes it. *YAP1* encodes a DNA-binding transcription factor belonging to the b-zip class of DNA-binding proteins. Overexpression of *YAP1* in yeast confers increased resistance to hydrogen peroxide, *o*-phenanthroline, heavy metals, and osmotic stress (*45*). We analyzed differential gene expression between a wild-type strain bearing a control plasmid and a strain with a plasmid expressing *YAP1* under the control of the strong *GAL1-10* promoter, both grown in galactose (that is, a condition that induces *YAP1* overexpression). Complementary DNA from the control and *YAP1* overexpressing strains, labeled with Cy3 and Cy5, respectively, was prepared from mRNA isolated from the two strains and hybridized to the microarray. Thus, red spots on the array represent genes that were induced in the strain overexpressing *YAP1*.

Of the 17 genes whose mRNA levels increased by more than threefold when

YAP1 was overexpressed in this way, five bear homology to aryl-alcohol oxidoreductases (Fig. 2 and Table 1). An additional four of the genes in this set also belong to the general class of dehydrogenases/oxidoreductases. Very little is known about the role of aryl-alcohol oxidoreductases in *S. cerevisiae*, but these enzymes have been isolated from ligninolytic fungi, in which they participate in coupled redox reactions, oxidizing aromatic, and aliphatic unsaturated alcohols to aldehydes with the production of hydrogen peroxide (*46, 47*). The fact that a remarkable fraction of the targets identified in this experiment belong to the same small, functional group of oxidoreductases suggests that these genes

might play an important protective role during oxidative stress. Transcription of a small number of genes was reduced in the strain overexpressing Yap1. Interestingly, many of these genes encode sugar permeases or enzymes involved in inositol metabolism.

We searched for Yap1-binding sites (TTACTAA or TGACTAA) in the sequences upstream of the target genes we identified (*48*). About two-thirds of the genes that were induced by more than threefold upon Yap1 overexpression had one or more binding sites within 600 bases upstream of the start codon (Table 1), suggesting that they are directly regulated by Yap1. The absence of canonical Yap1-bind-

Fig. 4. Coordinated regulation of functionally related genes. The curves represent the average induction or repression ratios for all the genes in each indicated group. The total number of genes in each group was as follows: ribosomal proteins, 112; translation elongation and initiation factors, 25; tRNA synthetases (excluding mitochondial synthetases), 17; glycogen and trehalose synthesis and degradation, 15; cytochrome c oxidase and reductase proteins, 19; and TCA- and glyoxylate-cycle enzymes, 24.

Table 1. Genes induced by *YAP1* overexpression. This list includes all the genes for which mRNA levels increased by more than twofold upon *YAP1* overexpression in both of two duplicate experiments, and for which the average increase in mRNA level in the two experiments was greater than threefold (*50*). Positions of the canonical Yap1 binding sites upstream of the start codon, when present, and the average fold-increase in mRNA levels measured in the two experiments are indicated.

ORF	Distance of Yap1 site from ATG	Gene	Description	Fold-increase
YNL331C			Putative aryl-alcohol reductase	12.9
YKL071W	162–222 (5 sites)		Similarity to bacterial csgA protein	10.4
YML007W		YAP1	Transcriptional activator involved in oxidative stress response	9.8
YFL056C	223, 242		Homology to aryl-alcohol dehydrogenases	9.0
YLL060C	98		Putative glutathione transferase	7.4
YOL165C	266		Putative aryl-alcohol dehydrogenase (NADP+)	7.0
YCR107W			Putative aryl-alcohol reductase	6.5
YML116W	409	ATR1	Aminotriazole and 4-nitroquinoline resistance protein	6.5
YBR008C	142, 167, 364		Homology to benomyl/methotrexate resistance protein	6.1
YCLX08C			Hypothetical protein	6.1
YJR155W			Putative aryl-alcohol dehydrogenase	6.0
YPL171C	148, 212	OYE3	NAPDH dehydrogenase (old yellow enzyme), isoform 3	5.8
YLR460C	167, 317		Homology to hypothetical proteins YCR102c and YNL134c	4.7
YKR076W	178		Homology to hypothetical protein YMR251w	4.5
YHR179W	327	OYE2	NAD(P)H oxidoreductase (old yellow enzyme), isoform 1	4.1
YML131W	507		Similarity to *A. thaliana* zeta-crystallin homolog	3.7
YOL126C		MDH2	Malate dehydrogenase	3.3

ing sites upstream of the others may reflect an ability of Yap1 to bind sites that differ from the canonical binding sites, perhaps in cooperation with other factors, or less likely, may represent an indirect effect of Yap1 overexpression, mediated by one or more intermediary factors. Yap1 sites were found only four times in the corresponding region of an arbitrary set of 30 genes that were not differentially regulated by Yap1.

Use of a DNA microarray to characterize the transcriptional consequences of mutations affecting the activity of regulatory molecules provides a simple and powerful approach to dissection and characterization of regulatory pathways and net-

works. This strategy also has an important practical application in drug screening. Mutations in specific genes encoding candidate drug targets can serve as surrogates for the ideal chemical inhibitor or modulator of their activity. DNA microarrays can be used to define the resulting signature pattern of alterations in gene expression, and then subsequently used in an assay to screen for compounds that reproduce the desired signature pattern.

DNA microarrays provide a simple and economical way to explore gene expression patterns on a genomic scale. The hurdles to extending this approach to any other organism are minor. The equipment

required for fabricating and using DNA microarrays (9) consists of components that were chosen for their modest cost and simplicity. It was feasible for a small group to accomplish the amplification of more than 6000 genes in about 4 months and, once the amplified gene sequences were in hand, only 2 days were required to print a set of 110 microarrays of 6400 elements each. Probe preparation, hybridization, and fluorescent imaging are also simple procedures. Even conceptually simple experiments, as we described here, can yield vast amounts of information. The value of the information from each experiment of this kind will progressively increase as more is learned about the functions of each gene and as additional experiments define the global changes in gene expression in diverse other natural processes and genetic perturbations. Perhaps the greatest challenge now is to develop efficient methods for organizing, distributing, interpreting, and extracting insights from the large volumes of data these experiments will provide.

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8. Primers for each known or predicted protein coding sequence were supplied by Research Genetics. PCR was performed with the protocol supplied by Research Genetics, using genomic DNA from yeast strain S288C as a template. Each PCR product was verified by agarose gel electrophoresis and was deemed correct if the lane contained a single band of appropriate mobility. Failures were marked as such in the database. The overall success rate for a single-pass amplification of 6116 ORFs was ~94.5%.
9. Glass slides (Gold Seal) were cleaned for 2 hours in a solution of 2 N NaOH and 70% ethanol. After rinsing in distilled water, the slides were then treated with a 1:5 dilution of poly-L-lysine adhesive solution (Sigma) for 1 hour, and then dried for 5 min at 40°C in a vacuum oven. DNA samples from 100-µl PCR reactions were purified by ethanol purification in 96-well microtiter plates. The resulting precipitates were resuspended in 3× standard saline citrate (SSC) and transferred to new plates for arraying. A custom-built arraying robot was used to print on a batch of 110 slides. Details of the design of the microarrayer are available at cmgm.stanford.edu/pbrown. After printing, the microarrays were rehydrated for 30 s in a humid chamber and then snap-dried for 2 s on a hot plate (100°C). The DNA was then ultraviolet (UV)-crosslinked to the surface by subjecting the slides to 60 mJ of energy (Stratagene Stratalinker). The rest of the poly-L-lysine surface was blocked by a 15-min incubation in a solution of 70 mM succinic anhydride dissolved in a solution consisting of 315 ml of 1-methyl-2-pyrrolidinone (Aldrich) and 35 ml of 1 M boric acid (pH 8.0). Directly after the blocking reac-

Time (hours)

Fig. 5. Distinct temporal patterns of induction or repression help to group genes that share regulatory properties. (A) Temporal profile of the cell density, as measured by OD at 600 nm and glucose concentration in the media. (B) Seven genes exhibited a strong induction (greater than ninefold) only at the last timepoint (20.5 hours). With the exception of IDP2, each of these genes has a CSRE UAS. There were no additional genes observed to match this profile. (C) Seven members of a class of genes marked by early induction with a peak in mRNA levels at 18.5 hours. Each of these genes contain STRE motif repeats in their upstream promoter regions. (D) Cytochrome c oxidase and ubiquinol cytochrome c reductase genes. Marked by an induction coincident with the diauxic shift, each of these genes contains a consensus binding motif for the HAP2,3,4 protein complex. At least 17 genes shared a similar expression profile. (E) SAM1, GPP1, and several genes of unknown function are repressed before the diauxic shift, and continue to be repressed upon entry into stationary phase. (F) Ribosomal protein genes comprise a large class of genes that are repressed upon depletion of glucose. Each of the genes profiled here contains one or more RAP1-binding motifs upstream of its promoter. RAP1 is a transcriptional regulator of most ribosomal proteins.

tion, the bound DNA was denatured by a 2-min incubation in distilled water at ~95°C. The slides were then transferred into a bath of 100% ethanol at room temperature, rinsed, and then spun dry in a clinical centrifuge. Slides were stored in a closed box at room temperature until used.

10. YPD medium (8 liters), in a 10-liter fermentation vessel, was inoculated with 2 ml of a fresh overnight culture of yeast strain DBY7286 (MATa, ura3, GAL2). The fermentor was maintained at 30°C with constant agitation and aeration. The glucose content of the media was measured with a UV test kit (Boehringer Mannheim, catalog number 716251) Cell density was measured by OD at 600-nm wavelength. Aliquots of culture were rapidly withdrawn from the fermentation vessel by peristaltic pump, spun down at room temperature, and then flash frozen with liquid nitrogen. Frozen cells were stored at −80°C.

11. Cy3-dUTP or Cy5-dUTP (Amersham) was incorporated during reverse transcription of 1.25 µg of polyadenylated [poly(A)+] RNA, primed by a dT(16) oligomer. This mixture was heated to 70°C for 10 min, and then transferred to ice. A premixed solution, consisting of 200 U Superscript II (Gibco), buffer, deoxyribonucleoside triphosphates, and fluorescent nucleotides, was added to the RNA. Nucleotides were used at these final concentrations: 500 µM for dATP, dCTP, and dGTP and 200 µM for dTTP. Cy3-dUTP and Cy5-dUTP were used at a final concentration of 100 µM. The reaction was then incubated at 42°C for 2 hours. Unincorporated fluorescent nucleotides were removed by first diluting the reaction mixture with of 470 µl of 10 mM tris-HCl (pH 8.0)/1 mM EDTA and then subsequently concentrating the mix to ~5 µl, using Centricon-30 microconcentrators (Amicon).

12. Purified, labeled cDNA was resuspended in 11 µl of 3.5× SSC containing 10 µg poly(dA) and 0.3 µl of 10% SDS. Before hybridization, the solution was boiled for 2 min and then allowed to cool to room temperature. The solution was applied to the microarray under a cover slip, and the slide was placed in a custom hybridization chamber which was subsequently incubated for ~8 to 12 hours in a water bath at 62°C. Before scanning, slides were washed in 2× SSC, 0.2% SDS for 5 min, and then 0.05× SSC for 1 min. Slides were dried before scanning by centrifugation at 500 rpm in a Beckman CS-6R centrifuge.

13. The complete data set is available on the Internet at cmgm.stanford.edu/pbrown/explore/index.html

14. For 95% of all the genes analyzed, the mRNA levels measured in cells harvested at the first and second interval after inoculation differed by a factor of less than 1.5. The correlation coefficient for the comparison between mRNA levels measured for each gene in these two different mRNA samples was 0.98. When duplicate mRNA preparations from the same cell sample were compared in the same way, the correlation coefficient between the expression levels measured for the two samples by comparative hybridization was 0.99.

15. The numbers and identities of known and putative genes, and their homologies to other genes, were gathered from the following public databases: Saccharomyces Genome Database (genome-www. stanford.edu), Yeast Protein Database (quest7. proteome.com), and Munich Information Centre for Protein Sequences (speedy.mips.biochem.mpg.de/ mips/yeast/index.htmlx).

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38. Cross-hybridization of highly related sequences can also occasionally obscure changes in gene expression, an important concern where members of gene families are functionally specialized and differentially regulated. The major alcohol dehydrogenase genes, ADH1 and ADH2, share 88% nucleotide identity. Reciprocal regulation of these genes is an important feature of the diauxic shift, but was not observed in this experiment, presumably because of cross-hybridization of the fluorescent cDNAs representing these two genes. Nevertheless, we were able to detect differential expression of closely related isoforms of other enzymes, such as HXK1/HXK2 (77% identical) [P. Herrero et al., Yeast 11, 137 (1995)], MLS1/DAL7 (73% identical) (20), and PGM1/PGM2 (72% identical) [D. Oh, J. E. Hopper, Mol. Cell. Biol. 10, 1415 (1990)], in accord with previous studies. Use in the microarray of deliberately selected DNA sequences corresponding to the most divergent segments of homologous genes, in lieu of the complete gene sequences, should relieve this problem in many cases.

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dance between the sets of genes that appeared to be induced was very high between the two experiments. When only the 355 genes that showed at least a twofold increase in mRNA in the tup1Δ strain in either of the duplicate experiments were compared, the correlation coefficient was 0.82.

42. The tup1Δ mutation consists of an insertion of the LEU2 coding sequence, including a stop codon, between the ATG of TUP1 and an Eco R I site 124 base pairs before the stop codon of the TUP1 gene.

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49. Microarrays were scanned using a custom-built scanning laser microscope built by S. Smith with software written by N. Ziv. Details concerning scanner design and construction are available at cmgm. stanford.edu/pbrown. Images were scanned at a resolution of 20 µm per pixel. A separate scan, using the appropriate excitation line, was done for each of the two fluorophores used. During the scanning process, the ratio between the signals in the two channels was calculated for several array elements containing total genomic DNA. To normalize the two channels with respect to overall intensity, we then adjusted photomultiplier and laser power settings such that the signal ratio at these elements was as close to 1.0 as possible. The combined images were analyzed with custom-written software. A bounding box, fitted to the size of the DNA spots in each quadrant, was placed over each array element. The average fluorescent intensity was calculated by summing the intensities of each pixel present in a bounding box, and then dividing by the total number of pixels. Local area background was calculated for each array element by determining the average fluorescent intensity for the lower 20% of pixel intensities. Although this method tends to underestimate the background, causing an underestimation of extreme ratios, it produces a very consistent and noise-tolerant approximation. Although the analog-to-digital board used for data collection possesses a wide dynamic range (12 bits), several signals were saturated (greater than the maximum signal intensity allowed) at the chosen settings. Therefore, extreme ratios at bright elements are generally underestimated. A signal was deemed significant if the average intensity after background subtraction was at least 2.5-fold higher than the standard deviation in the background measurements for all elements on the array.

50. In addition to the 17 genes shown in Table 1, three additional genes were induced by an average of more than threefold in the duplicate experiments, but in one of the two experiments, the induction was less than twofold (range 1.6- to 1.9-fold)

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Drug target validation and identification of secondary drug target effects using DNA microarrays

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We describe here a method for drug target validation and identification of secondary drug target effects based on genome-wide gene expression patterns. The method is demonstrated by several experiments, including treatment of yeast mutant strains defective in calcineurin, immunophilins or other genes with the immunosuppressants cyclosporin A or FK506. Presence or absence of the characteristic drug 'signature' pattern of altered gene expression in drug-treated cells with a mutation in the gene encoding a putative target established whether that target was required to generate the drug signature. Drug dependent effects were seen in 'targetless' cells, showing that FK506 affects additional pathways independent of calcineurin and the immunophilins. The described method permits the direct confirmation of drug targets and recognition of drug-dependent changes in gene expression that are modulated through pathways distinct from the drug's intended target. Such a method may prove useful in improving the efficiency of drug development programs.

Good drugs are potent and specific; that is, they must have strong effects on a specific biological pathway and minimal effects on all other pathways. Confirmation that a compound inhibits the intended target (drug target validation) and the identification of undesirable secondary effects are among the main challenges in developing new drugs. Comprehensive methods that enable researchers to determine which genes or activities are affected by a given drug might improve the efficiency of the drug discovery process by quickly identifying potential protein targets, or by accelerating the identification of compounds likely to be toxic. DNA microarray technology, which permits simultaneous measurement of the expression levels of thousands of genes, provides a comprehensive framework to determine how a compound affects cellular metabolism and regulation on a genomic scale[1-11]. DNA microarrays that contain essentially every open reading frame (ORF) in the *Saccharomyces cerevisiae* genome have already been used successfully to explore the changes in gene expression that accompany large changes in cellular metabolism or cell cycle progression[7-10].

In the modern drug discovery paradigm, which typically begins with the selection of a single molecular target, the ideal inhibitory drug is one that inhibits a single gene product so completely and so specifically that it is as if the gene product were absent. Treating cells with such a drug should induce changes in gene expression very similar to those resulting from deleting the gene encoding the drug's target. Here we have compared the genome-wide effects on gene expression that result from deletions of various genes in the budding yeast *S. cerevisiae* to the effects on gene expression that result from treatment

with known inhibitors of those gene products. Using the calcineurin signaling pathway as a model system, we tested an approach that permits identification of genes that encode proteins specifically involved in pathways affected by a drug. The FK506 characteristic pattern, or 'signature', of altered gene expression was not observed in mutant cells lacking proteins inhibited by FK506 (for example, a calcineurin or FK506-binding-protein mutant strain), but was observed in mutants deleted for genes in pathways unrelated to FK506 action (for example, a cyclophilin mutant strain). Conversely, the cyclosporin A (CsA) signature was not observed in CsA-treated calcineurin or cyclophilin mutant strains, but was seen in an FK506-binding-protein mutant strain treated with CsA. The method also demonstrates that FK506, a clinically used immunosuppressant, has 'off-target' effects that are independent of its binding to immunophilins. Thus, the approach we describe may provide a way to identify the pathways altered by a drug and to detect drug effects mediated through unintended targets.

Null mutants phenocopy drug-treated cells on a genomic scale

To test whether a null mutation in a drug target serves as a model of an ideal inhibitory drug, we examined the effects on gene expression associated with pharmacological or genetic inhibition of calcineurin function. Calcineurin is a highly conserved calcium- and calmodulin-activated serine/threonine protein phosphatase implicated in diverse processes dependent on calcium signaling[12-13]. In budding yeast, calcineurin is required for intracellular ion homeostasis[14], for adaptation to prolonged mating pheromone treatment[15] and in the regulation of

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Fig. 1 Model of antagonism of the calcineurin signaling pathway mediated by FK506 and cyclosporin A (CsA). Calcineurin activity is composed of a catalytic subunit (calcineurin A, encoded in yeast by the *CNA1* and *CNA2* genes), and calcium-binding regulatory subunits calmodulin (CMD) and calcineurin B (CnB). After entering cells, FK506 and CsA specifically bind and inhibit the peptidyl-proline isomerase activity of their respective immunophilins, FK506 binding proteins (FKBP) and cyclophilins (CyP). The most abundant immunophilins in yeast (Fpr1 and Cph1) are thought to mediate calcineurin inhibition. Drug–immunophilin complexes bind and inhibit the calcium- and calmodulin-stimulated phosphatase calcineurin. Among the substrates of calcineurin are transcriptional activators that act to modulate gene expression.
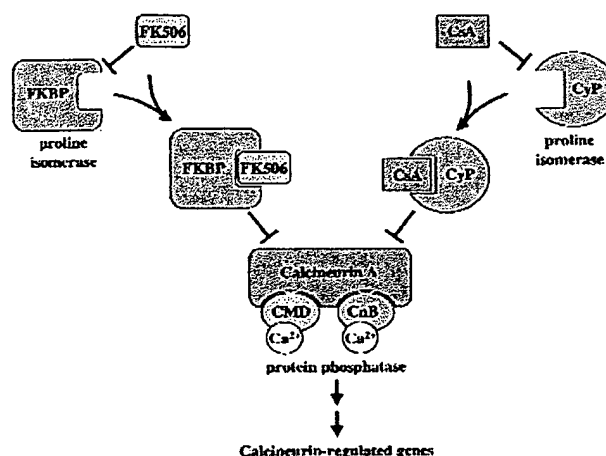
the onset of mitosis[16]. In mammals, calcineurin has been implicated in T-cell activation[12], in apoptosis[17], in cardiac hypertrophy[18] and in the transition from short-term to long-term memory[19]. In both organisms, calcineurin activity is inhibited by FK506 and CsA, immunosuppressant drugs whose effects on calcineurin are mediated through families of intracellular receptor proteins called immunophilins[12,20] (Fig. 1). To assess the effects of pharmacologic inhibition of calcineurin, wild-type *S. cerevisiae* was grown to early logarithmic phase in the presence or absence of FK506 or CsA. Isogenic cells, from which the genes encoding the catalytic subunits of calcineurin (*CNA1* and *CNA2*) had been deleted[21] (referred to as the *cna* or calcineurin mutant), were grown in parallel, in the absence of the drug. Fluorescently-labeled cDNA was prepared by reverse transcription of polyA+ RNA in the presence of Cy3- or Cy5-deoxynucleotide triphosphates and then hybridized to a microarray containing more than 6,000 DNA probes representing 97% of the known or predicted ORFs in the yeast genome. Simultaneous hybridization of Cy5-labeled cDNA from mock-treated cells and Cy3-labeled cDNA from cells treated with 1 μg/ml FK506 allowed the effect of drug treatment on mRNA levels of each ORF to be determined (Fig. 2a and b and data not shown). Similarly, effects of the calcineurin mutations on the mRNA levels of each gene were assessed by simultaneous hybridization of Cy5-labeled cDNA from wild-type cells and Cy3-labeled cDNA from the calcineurin mutant strain(Fig. 2c). For each comparison of this kind, reported expression ratios are the average of at least two hybridizations in which the Cy3 and Cy5 fluors were reversed to remove biases that may be introduced by gene-specific differences in incorporation of the two fluors (data not shown).

Treatment with FK506 in these growth conditions resulted in a signature pattern of altered gene expression in which mRNA levels of 36 ORFs changed by more than twofold (http://www.rosetta.org). A very similar pattern of altered gene expression was observed when the calcineurin mutant strain was compared to wild-type cells. Comparison of the changes in mRNA expression of each gene resulting from treatment of wild-type cells with FK506 with mRNA expression changes resulting from deletion of the calcineurin genes showed the considerable similarity of the global transcript alterations in response to the two perturbations (Fig. 2b–d). Quantification of this similarity using the correlation coefficient (ρ) showed large correlations between the FK506 treatment signature and the calcineurin deletion signature (ρ = 0.75 ± 0.03), as well as the CsA treatment signature (ρ = 0.94±0.02), but not with a randomly selected deletion mutant strain (deleted for the *YER071C* gene; ρ = –0.07 ± 0.04; Fig. 2e). The FK506 treatment signature was also compared with those of more than 40 other deletion mutant strains or drug-treatments thought to affect

unrelated pathways, and none had statistically significant correlations. These data establish that genetic disruption of calcineurin function provides a close and specific phenocopy of treatment with FK506 or CsA.

To avoid generalizing from a single example, we also compared the effects of treatment of wild-type cells with 3-aminotriazole (3-AT) with the effects of deletion of the *HIS3* gene. *HIS3* encodes imidazoleglycerol phosphate dehydratase, which catalyzes the seventh step of the histidine biosynthetic pathway in yeast[22]; 3-AT is a competitive inhibitor of this enzyme that triggers a large transcriptional amino-acid starvation response[23]. Microarray analysis of wild-type and isogenic *his3*-deficient strains demonstrated the expected large genome-wide transcriptional responses (involving more than 1,000 ORFs) resulting from treatment with 3-AT (Fig. 3a) or from *HIS3* deletion (Fig. 3c). Quantitative comparison of the 3-AT treatment signature and the *his3* mutant signature showed a high level of correlation (ρ= 0.76 ± 0.02) that even extended to genes that experienced small changes in expression level (Fig. 3b). As a negative control, the correlations between the 3-AT treatment signature or the *his3* mutant signature and the calcineurin mutant strain were not statistically significant (ρ = 0.09 ± 0.06 and –0.01 ± 0.04, respectively). That both the calcineurin/FK506 and the *his3*/3-AT comparisons were highly correlated indicates that in many cases the expression profile resulting from a gene deletion closely resembles the expression profile of wild-type cells treated with an inhibitor of that gene's product.

'Decoder' strategy: Drug target validation with deletion mutants

Because pharmacological inhibition of different targets might give similar or identical expression profiles, simple comparison of drug signatures to mutant signatures is unlikely to unambiguously identify a drug's target. To overcome this limitation, an additional 'decoder' step is used. We first compare the expression profile of wild-type drug-treated cells to the expression profiles from a panel of genetic mutant strains, using a correlation coefficient metric. Mutant strains whose expression profile is similar to that of drug-treated wild-type cells are selected and subjected to drug treatment, generating the drug signature in the mutant strain (that is, the mutant drug signature). If the mutated gene encodes a protein involved in a pathway affected by the drug, we expect the drug signature in mutant cells to be different (or absent, for an ideal drug) from the drug signature seen in wild-type cells.

Fig. 2 Expression profiles from FK506-treated wild-type (wt) cells and a calcineurin-disruption mutant strain share a genome-wide correlation. DNA microarray analysis showing changes in gene expression resulting from FK506 treatment (*a* and *b*) or from genetic disruption of genes encoding calcineurin (*c*). *a*, Pseudo-color image of the results of simultaneous hybridization of Cy5-labeled cDNA (red) from mock-treated strain R563 and Cy3-labeled cDNA (green) from strain R563 treated with 1 µg/ml FK506. *b*, Enlarged view of the boxed area in *a*. Arrowheads indicate specific ORFs induced or repressed. *c*, Pseudo-color image of the results of simultaneous hybridization of Cy5-labeled cDNA (red) from strain R563 and Cy3-labeled cDNA (green) from strain MCY300 (deleted for the *CNA1,CNA2* catalytic subunits of calcineurin). Arrows indicate specific ORFs induced or repressed. *d*, The $\log_{10}$ of the expression ratio for each ORF derived from the FK506 treatment hybridizations is plotted versus the $\log_{10}$ of the expression ratio in the calcineurin mutant hybridizations. ORFs that were induced or repressed in both experiments are shown as green and red dots, respectively. *e*, The $\log_{10}$ of the expression ratio for each ORF derived from the FK506 treatment hybridizations is plotted versus the $\log_{10}$
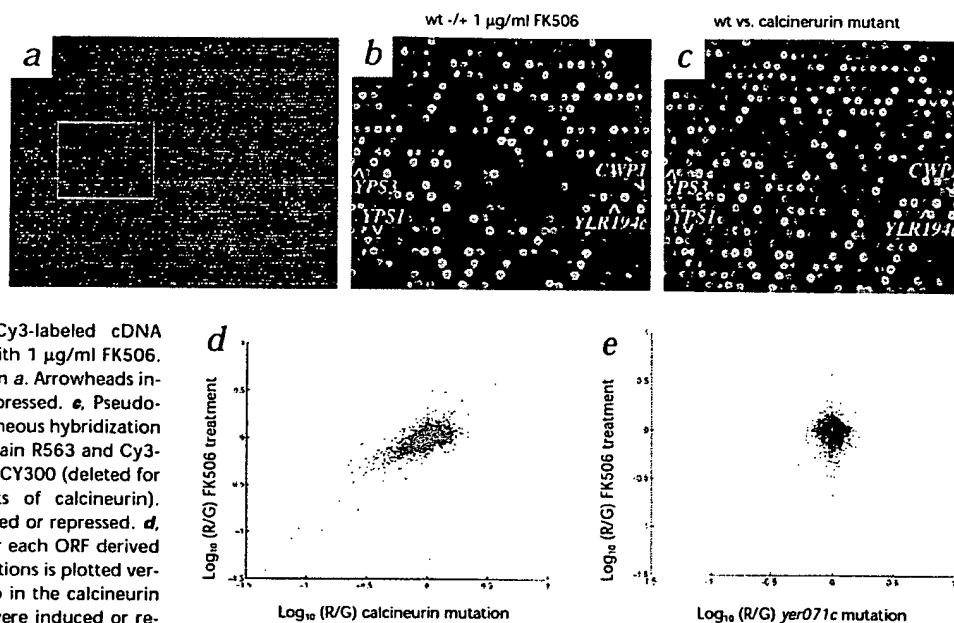
of the expression ratio in the *yer071c* mutant hybridizations. No ORFs were induced or repressed in both experiments.

To illustrate this, we treated the *his3* mutant strain with 3-AT. The signature pattern of altered gene expression resulting from treatment of the mutant strain with 3-AT was much less complex than that of the 3-AT signature in wild-type cells (Fig. 4). This is seen simply by examining plots of mean intensity of the hybridization signal (which approximately reflects level of expression) versus the expression ratio for each ORF (Fig. 4). Genes that were expressed at higher or lower levels in 3-AT treated cells or in *his3* mutant cells are shown as red and green dots, respectively. We analyzed the 3-AT signature in wild-type (Fig. 4a) and *his3* mutant cells (Fig. 4c), as well as the *his3* mutant strain signature (Fig. 4b). Whereas histidine limitation induced by 3-AT induced more than 1,000 transcription-level changes in the wild-type strain, few or no transcript level changes were induced by treatment of the *his3*-deletion strain with 3-AT. This indicates that with the growth conditions used, essentially all of the effects of 3-AT depend on or are mediated through the HIS3 gene product.

Applying this approach to the calcineurin signaling pathway showed the specificity of the method. The calcineurin mutant strain and strains with deletions in the genes encoding the most abundant immunophilins in yeast[12] (*CPH1* and *FPR1*) were treated with either FK506 or CsA to determine the profiles

of altered gene expression resulting from drug treatment of the mutant cells (that is, mutant +/- drug). We compared the drug signatures in the mutants to the wild-type drug signature using the correlation coefficient metric (Table 1). Although the signature generated by treatment of wild-type cells with FK506 was highly correlated to the calcineurin mutant strain signature (ρ = 0.75 ± 0.03), it bore no similarity to the profile after treatment of the calcineurin mutant strain with FK506 (ρ = -0.01 ± 0.07). This indicates that FK506 was unable to elicit its normal transcriptional response in the calcineurin mutant strain. Likewise, treatment of the *fpr1* mutant strain with FK506 elicited an expression profile that was not correlated to the FK506 signature in the wild-type strain (ρ = -0.23 ± 0.07), indicating that the *FPR1* gene product is likely to be involved in the pathway affected by FK506. The same was true for the *cna fpr1* mutant strain. In contrast, treatment of the *cph1* mutant strain with FK506 generated an expression profile highly correlated with the wild-type FK506 expression profile (ρ = 0.79 ± 0.03), indicating the *cph1* mutation did not block the mode of action of FK506 and thus is not directly involved in the pathway affected by FK506. We tabulated the change in expression in response to FK506 in different mutant strains for all ORFs with expression ratios greater than 1.8 in FK506-treated cells or in the calcineurin mutant strain (Fig. 5a).The calcineurin mutant strain signature and the FK506 responses in wild-type and the *cph1* mutant strain are similar, and there are no transcript-level changes (seen in black) for treatment of the calcineurin, *fpr1* and *cna fpr1* mutant strains with FK506 (Fig. 5a).

Similar experiments and analyses with CsA provided further validation of this approach. The expression profile elicited by treatment of wild-type cells with CsA was highly corre-

Table 1 Signature correlation of expression ratios as a result of FK506 treatment in various mutant strains

	wild-type +/-FK506	cna +/-FK506	fpr1 +/-FK506	cna fpr1 +/-FK506	cph1 +/-FK506
wild-type +/- FK506	0.93 ± 0.04	-0.01 ± 0.07	-0.23 ± 0.07	0.12 ± 0.07	0.79 ± 0.03

Signature correlation shows the absence of the FK506 signature specifically in the calcineurin (*cna*) and *fpr1* (major FK506 binding protein) deletion mutants. *cna* represents the mutant with deletions of the catalytic subunits of calcineurin, *CNA1* and *CNA2*. The correlation coefficient reported in the first column represents the correlation between two pairs of hybridizations from independent wild-type +/- FK506 experiments.

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Fig. 3 Expression profiles from a *his3* mutant strain and wild-type (wt) cells treated with 3-AT share a genome-wide correlation. DNA microarray analysis showing changes in gene expression resulting from 3-AT treatment (*a*) or from genetic disruption of the *HIS3* gene (*c*). *a*, Pseudo-color image of the results of simultaneous hybridization of Cy5-labeled cDNA (red) from mock-treated wild-type strain R491 and Cy3-labeled cDNA (green) from strain R491 treated with 10 mM 3-AT. *b*, Plot of the log₁₀ of the expression ratio for each ORF derived from the 3-AT treatment hybridizations is plotted versus the log₁₀ of the expression ratio in the *his3* mutant hybridizations. ORFs that were induced or repressed in both experiments are shown as green and red dots, respectively. The correlation of expression ratios applies not only to genes with large expression ratios (for example, *CHA1* and *ARG1*), but also extends to genes with expression ratios less than 2 (for example, *ILV1* and *CPH1*). *ILV1* is induced 1.9-fold and 1.5-fold, and *CPH1* is downregulated 1.9-fold
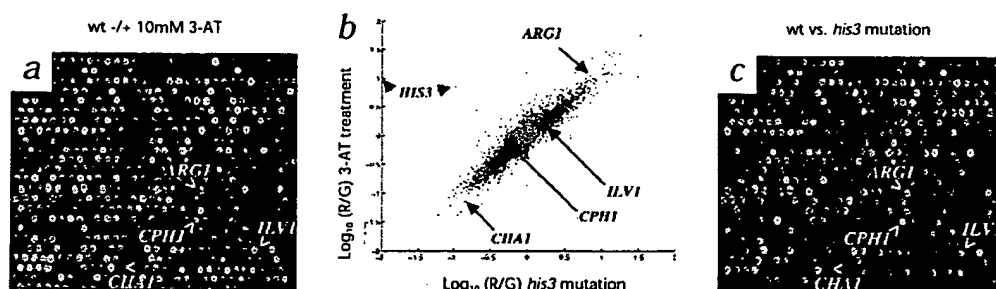
and 1.7-fold, in cells treated with 3-AT and *his3* mutant cells, respectively. Two ORFs do not fall on the line x = y. The leftmost point is the *HIS3* data point, which is induced by 3-AT treatment but which is not absent from the *his3* mutant strain. The other point is *YOR203w*. Both data points are labeled *HIS3* because hybridization to *YOR203w* is most likely due to *HIS3* mRNA, as *YOR203w* overlaps the *HIS3* open reading frame. *e*, Pseudo-color image of the results of simultaneous hybridization of Cy5-labeled cDNA (red) from wild-type strain R491 and Cy3-labeled cDNA (green) from strain R1226, deleted for the *HIS3* gene. Arrowheads indicate specific ORFs induced or repressed.

lated to the profile elicited by mutation of the calcineurin genes (ρ = 0.71 ± 0.04), but did not correlate with the expression profile resulting from treatment of the calcineurin mutant strain with CsA (ρ = –0.05 ± 0.07; Table 2), indicating that the genetic deletion of calcineurin interfered with the ability of CsA to elicit its normal transcriptional response. Likewise, the CsA signature was essentially absent in CsA-treated *cph1* mutant cells, and the expression profile of CsA-treated *cph1* mutant cells correlated poorly to that of CsA-treated wild-type cells (ρ = 0.18 ± 0.07). Thus, the *CPH1* gene product was required for the CsA response seen in wild-type cells. Conversely, treatment of *fpr1* mutant cells with CsA resulted in an expression pattern very similar to the profile of CsA-treated wild-type cells (ρ = 0.77 ± 0.03), indicating that *FPR1* was not necessary for the CsA-mediated effects. Analysis of individual ORFs affected by CsA and their expression ratios over the entire set of experiments confirmed that *CPH1* and the genes encoding calcineurin, but not

FPR1, are necessary for the wild-type CsA response (Fig. 5*b*). The observation that the profiles resulting from FK506 or CsA drug treatment are similar to that of the calcineurin deletion mutant strain might allow the prediction that calcineurin was involved in the pathway affected by these drugs. But because the expression profile of the *fpr1* mutant strain did not bear a strong similarity to the wild-type drug expression profile for FK506, it is obvious that the drug treatment of the mutant strains was necessary to identify Fpr1, but not Cph1, as a potential FK506 drug target. In the same way, the 'decoder' strategy was necessary to identify Cph1, but not Fpr1, as a potential drug target for CsA.

'Decoder' approach can identify secondary drug effects

For a drug that has a single biochemical target, the strategy outlined above may be useful in target validation. In many cases, however, a compound may affect multiple pathways and elicit a very complex signature. 'Decoding' such a complex signature
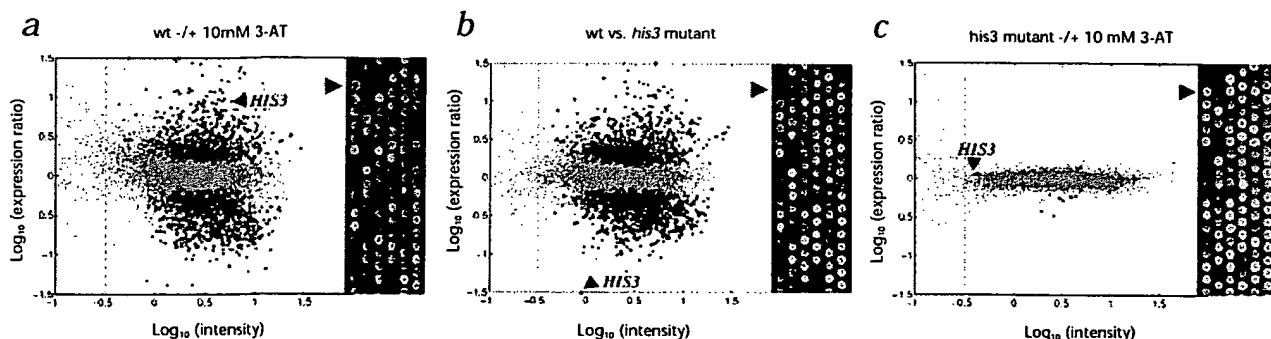
Fig. 4 Treatment of the *his3* mutant strain with 3-AT shows nearly complete loss of 3-AT signature. A plot of the log₁₀ of the mean intensity of hybridization for each ORF versus the log₁₀ of its expression ratio for each experiment is shown next to a pseudo-color image of a representative portion of the microarray. ORFs that are induced or repressed at the 95% confidence level are shown in green and red, respectively. *a*, Expression profile from treatment of the wild-type (wt) strain with 3-AT. Cy5-labeled cDNA (red) from mock-treated strain R491 and Cy3-labeled cDNA (green) from strain R491 treated with 10 mM 3-AT. *b*, Expression profile

from the *his3* deletion strain. Cy5-labeled cDNA (red) from strain R491 and Cy3-labeled cDNA (green) from strain R1226, deleted for the *HIS3* gene. *c*, Expression profile of treatment of the *his3* deletion strain with 3-AT. Cy3-labeled cDNA (red) from *his3*-deleted strain R1226 and Cy5-labeled cDNA (green) from strain R1226 treated with 10 mM 3-AT. Arrowheads indicate the DNA probe and data point corresponding to the *HIS3* gene. The blue dashed line represents the threshold below which errors tend to increase rapidly because spot intensities are not sufficiently above background intensity.

Table 2	Signature correlation of expression ratios as a result of CsA treatment in various mutant strains				
	wild-type +/−CsA	cna +/−CsA	fpr1 +/−CsA	cna cph1 +/−CsA	cph1 +/−CsA
wild-type +/− CsA	0.94 ± 0.04	−0.05 ± .07	0.77 ± 0.03	−0.11 ± 0.07	0.18 ± 0.07

Signature correlation shows the absence of the CsA signature specifically in the calcineurin (cna) and cph1 (cyclophilin) deletion mutants. cna represents the mutant with deletions of the catalytic subunits of calcineurin, CNA1 and CNA2. The correlation coefficient reported in the first column represents the correlation between two pairs of hybridizations from independent wild-type +/− CsA experiments.

into the effects mediated through the intended target (the 'on-target signature') and those mediated through unintended targets (the 'off-target' signature) might be useful in evaluating a compound's specificity. Our 'decoder' strategy is based on the premise that 'off-target' signature should be insensitive to the genetic disruption of the primary target.

To determine whether the 'decoder' approach could identify an 'off-target' profile, we looked for a drug-responsive gene whose expression is insensitive to deletion of the primary target. To increase the likelihood of observing such genes, the same strains described in Tables 1 and 2 were treated with higher concentrations (50 µg/ml) of FK506. This led to a much more complex expression profile in wild-type cells, indicating that at this higher concentration, FK506 was inhibiting or activating additional targets. Several of the ORFs in this expanded FK506-induced expression profile were not affected by the calcineurin, cph1 or fpr1 mutations, as drug treatment of these mutant strains did not block their presence in the FK506 expression signature (Fig. 6). This indicates that FK506 was triggering changes in transcript levels of many genes through pathways independent of calcineurin, CPH1 and FPR1. Many of the upregulated ORFs in the 'off-target' pathway were genes reported to be regulated by the transcriptional activator Gcn4 (ref. 24). In some strains, a reporter gene under GCN4 control was induced in response to FK506 treatment[25]. To determine whether GCN4 is involved in this pathway that is independent of calcineurin, CPH1 and FPR1, we analyzed the effects of treatment with high-dose FK506 on global gene expression in a strain with a GCN4 deletion (Fig. 6). Of the 41 ORFs with calcineurin-independent expression ratios greater than 4, 32 were not induced in the gcn4 mutant, indicating that their induction by FK506 was GCN4-dependent. Not all GCN4-regulated genes were induced by FK506. This FK506-induced subset of GCN4-regulated genes may be those most sensitive to subtle changes in Gcn4 levels, or perhaps other regulatory circuits prevent FK506 activation of some GCN4-regulated genes. Seven of the remaining nine ORFs induced by FK506 were independent of

both the calcineurin and GCN4 pathways. The simplest explanation is that FK506 inhibits or activates additional pathways. Members of this class include SNQ2 and PDR5, genes that encode drug efflux pumps with structural homology to mammalian multiple drug resistance proteins[26]. FK506 may interact directly with Pdr5 to inhibit its function[27]. Our results indicate that treatment with FK506 leads to four-fold-to-sixfold induction of PDR5 mRNA levels. YOR1, another gene that can confer drug resistance, is also induced threefold-to-fourfold by FK506. Thus, drug treatment of strains with mutations in the primary targets can prove useful in identifying effects mediated by secondary drug targets, including the nature and extent of newly discovered and previously unsuspected pathways affected by the drug.

We describe here a method for drug target validation and the identification of secondary drug target effects that uses DNA microarrays to survey the effects of drugs on global gene expression patterns. We established that genetic and pharmacologic inhibition of gene function can result in extremely similar changes in gene expression. We also demonstrated that one can confirm a potential drug target by treating a deletion mutant defective in the gene encoding the putative target. Drug-mediated signatures from strains with mutations in pathways or processes directly or indirectly affected by the drug bore little or
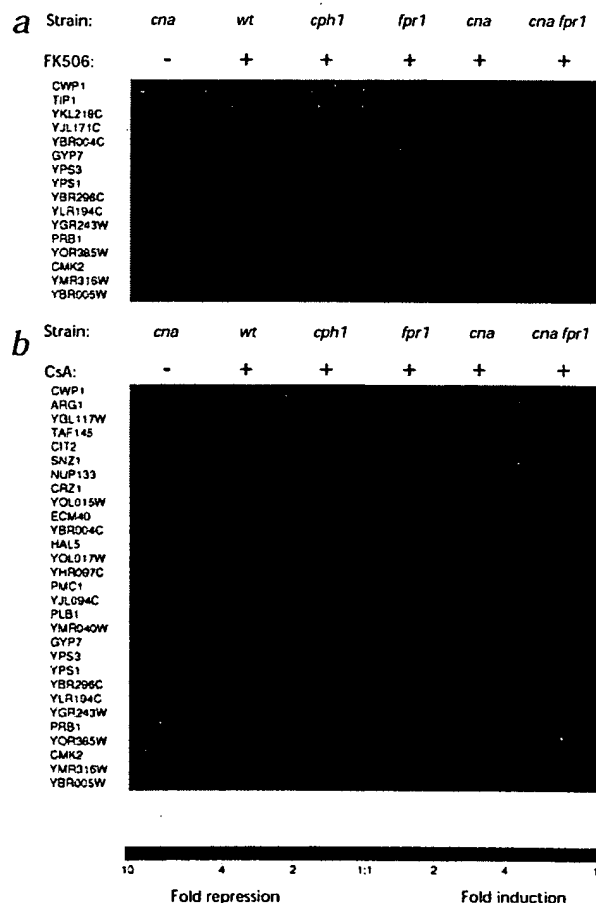
Fig. 5 Response of FK506 and CsA signature genes in strains with deletions in different genes. Genes with expression ratios greater than a factor of 1.8 in response to treatment with 1 µg/ml FK506 (a) or 50 µg/ml CsA (b) are listed (left side) and their expression ratios in the indicated strain are shown on the green (induction)–red (repression) color scale. a, Calcineurin (cna) mutant and FK506 treatment signature genes are in the first two columns. Almost all FK506 signature genes have expression ratios near unity in deletion strains involved in pathways affected by FK506 (calcineurin, fpr1 and cna fpr1 mutants) but not in deletion strains in unrelated pathways (cph1). b, Calcineurin (cna) mutant and CsA treatment signature genes are in the first two columns. Almost all CsA signature genes have expression ratios near unity in deletion strains involved in pathways affected by CsA (calcineurin, cph1 and cna cph1 mutants) but not in deletion strains in unrelated pathways (fpr1).

ARTICLES

Strain:	wt	cna	fpr1	cph1	cna fpr1	gen4
FK506:	+	+	+	+	+	+

CNA-dependent
YBR005W
YMR316W
CMK2

GCN4-dependent
MET16
CPA1
YOL119C
HOM2
YBR047W
SSU1
IDP1
ARG8
NIT1
YHM1
YGL184C
HAD1
CPA2
ADH5
YOL059W
YJR130C
ARO3
HIS3
PCL5
RIB5
SNO1
YHR029C
YBR147W
ARG3
HIS4
YGL117W
SNZ1
ARG5,6
ARG4
YPL250C
YER175C
ARG1

CNA- and GCN4-independent
URA1
HSC82
SSA2
GCV2
PDR5
SMC2
HIS5

Complex behavior class
YER024W
YMR085W

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10        4        2      1:1     2        4       10
     Fold repression              Fold induction
```

Fig. 6 Response of FK506 signature genes in strains with deletions in different genes. Genes with expression ratios greater than a factor of 4 in at least one experiment are listed and their expression ratios in the indicated strain are shown in the green (induction)–red (repression) color scale. The genes have been divided into classes corresponding to these expected behaviors: 'CNA-dependent' genes respond to FK506 (50 µg/ml) except when either calcineurin genes or FPR1 or both are deleted; 'GCN4-dependent' genes respond to FK506 except when GCN4 is deleted. These genes still respond to FK506 when calcineurin genes or FPR1 or CPH1 are deleted; that is, their responses are not mediated by calcineurin, Cph1, or Fpr1. 'CNA- and GCN4-independent' genes respond to FK506 in all deletion strains tested. A 'complex behavior' class is provided for those genes that did not match the model of FK506 response mediated through calcineurin or Fpr1 or separately through Gcn4.

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no similarity to the wild-type drug expression profile. In contrast, drug-mediated signatures from strains with mutations in genes involved in pathways unrelated to the drug's action showed extensive similarity to the wild-type drug signature. By applying this approach to a drug that affects multiple pathways (FK506), we were able to decode a complex signature into component parts, including the identification of an 'off-target' signature that was mediated through pathways independent of calcineurin or the Fpr1 immunophilin.

Discussion

It is well-established that high-throughput biochemical screening can identify potent inhibitory compounds against a given target. The 'decoder' approach described here complements this process by evaluating the equally important property of specificity: the tendency of a compound to inhibit pathways other than that of its intended target. The ability to observe such 'off-target' effects will likely be useful in several ways. Profiling compounds with known toxicities will allow the development of a database of expression changes associated with particular toxicities. Recognition of potential toxicities in the 'off-target' signatures of otherwise promising compounds then may allow earlier identification of those likely to fail in clinical trials. Comparing the extent and peculiarities of 'off-target' signatures of promising drug candiates could provide a new way to group compounds by their effects on secondary pathways, even before those effects are understood. This may prove to be an alternative, potentially more effective, way to select compounds for animal and clinical trials. Some drugs are more effective against a related protein than against the originally intended target. Sildenafil (Viagra™), for example, was initially developed as a phosphodiesterase inhibitor to control cardiac contractility, but was found to be highly specific for phosphodiesterase 5, an isozyme whose inhibition overcomes defects in

penile erection. It is possible that application of the 'decoder' to other compounds may show that they too have a potent activity against a target distinct from their intended target.

The ability to decode drug effects is dependent on the availability of functionally 'targetless' cells. In yeast, this is being achieved by systematically disrupting each yeast gene (Saccharomyces Deletion Consortium; http://sequence-www.stanford.edu/group/yeast_deletion_project/deletion.html). Efforts are underway to obtain expression profiles from each deletion mutant strain. Determining signatures resulting from inactivation of essential genes presents a unique problem, but it may be possible to do so by examining heterozygotes or by using a controllable promoter to reduce expression of the essential gene. Although it is already feasible to test several compounds in dozens of yeast strains, another challenge for the 'decoder' strategy will be the efficient selection of the mutants with deletions in genes most likely to encode the intended drug target. The signature correlation plots described are one metric that could be used as part of that selection process, but others need to be explored. Applying the 'decoder' to mammalian cells presents additional challenges. It is considerably more difficult to isolate functionally 'targetless' cells. Strategies involving titratable promoters, known specific inhibitors, anti-sense RNAs, ribozymes, and methods of targeting specific proteins for degradation are possible and should be tested. Another limitation is that not all cell types express the same set of genes and therefore 'off-target' effects may be different in different cell types. In addition, applying the 'decoder' to human cells will also require technical improvements that allow expression profiling from a small number of cells. Even the broader question of whether the insensitivity of 'off-target' signatures to the disruption of the main target is the exception or the rule can only be answered by the accumulation of more data. Barkai and Leibler, however, have argued in favor of robustness of biological networks, indicating that drug perturbations ('off-target' signatures) may be robust even when the system is subjected to another perturbation (such as a genetic disruption)(ref. 28). Many practical developments will be necessary if the 'decoder' concept is to be broadly applied.

Expression arrays have been used mainly as an initial screen for genes induced in a particular tissue or process of interest by focusing on genes with large expression ratios. We have found, however, that effort to refine experimental protocols and repeat experiments increases the reliability of the data and permits new applications. For example, it provides a larger set

Table 3   Yeast strains used

Strain	Relevant genotype	Reference
YPH499	Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1	(34)
R563	Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 his3::HIS3	(this study)
R558	Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 fpr1::HIS3	(this study)
R567	Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cph1::HIS3	(this study)
MCY300	Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cna1Δ1::hisG cna2Δ1::HIS3	(21)
R132	Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cna1Δ1::hisG cna2Δ1::HIS3 cph1::kanʳ	(this study)
R133	Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cna1Δ1::hisG cna2Δ1::HIS3 fpr1::kanʳ	(this study)
R559	Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 his3::HIS3 gcn4::LEU2	(this study)
BY4719	Mata trp1-Δ63 ura3-Δ0	(35)
BY4738	Matα trp1-Δ 63 ura3-Δ0	(35)
R491	Mata/α BY4719 X BY4738	(this study)
BY4728	Mata his3-Δ200 trp1-Δ63 ura3-Δ0	(35)
BY4729	Matα his3-Δ 200 trp1-Δ63 ura3-Δ0	(35)
R1226	Mata/α BY4728 X BY4729	(this study)

of genes at higher confidence levels that serve as a more unique signature for a given protein perturbation. In addition, it allows subtle signatures to be detected, when, for example, a protein is only partially inhibited. This may enable clinical monitoring of small changes in protein function in disease or toxicity states before they could otherwise be detected. Because the functions of many genes detected on transcript arrays are known, these microarrays are powerful tools that provide detailed information about a cell's physiology. For example, changes in the flux through a metabolic pathway are reflected in transcriptional changes in genes in the pathway[7]. Furthermore, it may be possible to indirectly measure protein activity levels from expression profiling data (S.F., et al., unpublished data). Thus, although the eventual development of genomic methods allowing the direct measurement of all cellular protein levels will be an important achievement, transcript array technology offers an immediate and robust means of evaluating the effects of various treatments on gene expression and protein function.

Methods

Construction, growth and drug treatment of yeast strains. The strains used in this study (Table 3) were constructed by standard techniques[29]. To construct strain R559, strain R563 was transformed to Leu⁺ with plasmid pM12 digested by SaI and MluI (provided by A. Hinnebusch and T. Dever). Strains R132 and R133 were constructed by transforming the bacterial kanamycin resistance cassette[30] flanked by genomic DNA from the CPH1 and FPR1 loci, respectively, and selecting for G418-resistant colonies. For experiments with FK506, cells were grown for three generations to a density of 1×10^7 cells/ml in YAPD medium (YPD plus 0.004% adenine) supplemented with 10 mM calcium chloride as described[31]. Where indicated, FK506 was added to a final concentration of 1 μg/ml 0.5 h after inoculation of the culture or to 50 μg/ml 1 h before cells were collected. CsA was used at a final concentration of 50 μg/ml. Cells were broken by standard procedures[32] with the following modifications: Cell pellets were resuspended in breaking buffer (0.2 M Tris HCl pH 7.6, 0.5 M NaCl, 10 mM EDTA, 1% SDS), vortexed for 2 min on a VWR multi-tube vortexer at setting 8 in the presence of 60% glass beads (425–600 μm mesh; Sigma) and phenol:chloroform (50:50, volume/volume). After separation of the phases, the aqueous phase was re-extracted and ethanol-precipitated. Poly A⁺ RNA was isolated by two sequential chromatographic purifications over oligo dT cellulose (New England Biolabs, Beverly, Massachusetts) using established protocols[32].

For experiments using 3-AT, wild-type or his3/his3 cells were grown to early logarithmic phase in SC medium, pelleted and resuspended in SC medium lacking histidine for 1 hr in the presence or absence of 10 mM 3-AT, as indicated. Cells were harvested and mRNA isolated as above. FK506 was obtained from the Swedish Hospital Pharmacy (Seattle, Washington) and purified to homogeneity by ethyl acetate extraction by J. Simon (Fred Hutchinson Cancer Research Center, Seattle, Washington). CsA was obtained from Alexis Biochemicals (San Diego, California); 3-AT was from Sigma.

Preparation and hybridization of the labeled sample. Fluorescently-labeled cDNA was prepared, purified and hybridized essentially as described[7]. Cy3- or Cy5-dUTP (Amersham) was incorporated into cDNA during reverse transcription (Superscript II; Life Technologies) and purified by concentrating to less than 10 μl using Microcon-30 microconcentrators (Amicon, Houston, Texas). Paired cDNAs were resuspended in 20–26 μl hybridization solution (3 × SSC, 0.75 μg/ml polyA DNA, 0.2% SDS) and applied to the microarray under a 22- × 30-mm coverslip for 6 h at 63 °C, all according to a published method[7].

Fabrication and scanning of microarrays. PCR products containing common 5′ and 3′ sequences (Research Genetics, Huntsville, Alabama) were used as templates with amino-modified forward primer and unmodified reverse primers to PCR amplify 6,065 ORFs from the S. cerevisiae genome. Our first-pass success rate was 94%. Amplification reactions that gave products of unexpected sizes were excluded from subsequent analysis. ORFs that could not be amplified from purchased templates were amplified from genomic DNA. DNA samples from 100-μl reactions were isopropanol-precipitated, resuspended in water, brought to a final concentration of 3× SSC in a total volume of 15 μl, and transferred to 384-well microtiter plates (Genetix Limited, Christchurch, Dorset, England). PCR products were spotted onto 1 × 3-inch polylysine-treated glass slides by a robot built essentially according to defined specifications[1,5,7] (http://cmgm.stanford.edu/pbrown/MGuide). After being printed, slides were processed according to published protocols[7].

Microarrays were imaged on a prototype multi-frame CCD camera in development at Applied Precision (Issaquah, Washington). Each CCD image frame was approximately 2-mm square. Exposure times of 2 s in the Cy5 channel (white light through Chroma 618–648 nm excitation filter, Chroma 657–727 nm emission filter) and 1 s in the Cy3 channel (Chroma 535–560 nm excitation filter, Chroma 570–620 nm emission filter) were done consecutively in each frame before moving to the next, spatially contiguous frame. Color isolation between the Cy3 and Cy5 channels was about 100:1 or better. Frames were 'knitted' together in software to make the complete images. The intensity of spots (about 100 μm) were quantified from the 10-μm pixels by frame-by-frame background subtraction and intensity averaging in each channel. Dynamic range of the resulting spot intensities was typically a ratio of 1,000 between the brightest spots and the background-subtracted additive error level. Normalization between the channels was accomplished by normalizing each channel to the mean intensities of all genes. This procedure is nearly equivalent to normalization between channels using the intensity

ratio of genomic DNA spots[7], but is possibly more robust, as it is based on the intensities of several thousand spots distributed over the array.

Signature correlation coefficients and their confidence limits. Correlation coefficients between the signature ORFs of various experiments were calculated using:

$$\rho = \sum_k x_k y_k \ / \ (\sum_k x_k^2 \sum_k y_k^2)^{1/2}$$

where x_k is the $\log_{10}$ of the expression ratio for the k^{th} gene in the x signature, and y_k is the $\log_{10}$ of the expression ratio for the k^{th} gene in the y signature. The summation is over those genes that were either up- or down-regulated in either experiment at the 95% confidence level. These genes each had a less than 5% chance of being actually unregulated (having expression ratios departing from unity due to measurement errors alone). This confidence level was assigned based on an error model which assigns a lognormal probability distribution to each gene's expression ratio with characteristic width based on the observed scatter in its repeated measurements (repeated arrays at the same nominal experimental conditions) and on the individual array hybridization quality. This latter dependence was derived from control experiments in which both Cy3 and Cy5 samples were derived from the same RNA sample. For large numbers of repeated measurements the error reduces to the observed scatter. For a single measurement the error is based on the array quality and the spot intensity.

Random measurement errors in the x and y signatures tend to bias the correlation towards zero. In most experiments, most genes are not significantly affected but do show small random measurement errors. Selecting only the '95% confidence' genes for the correlation calculation, rather than the entire genome, reduces this bias and makes the actual biological correlations more apparent.

Correlations between a profile and itself are unity by definition. Error limits on the correlation are 95% confidence limits based on the individual measurement error bars, and assuming uncorrelated errors[33]. They do not include the bias mentioned above; thus, a departure of ρ from unity does not necessarily mean that the underlying biological correlation is imperfect. However, a correlation of 0.7 ± 0.1, for example, is very significantly different from zero. Small (magnitude of $\rho < 0.2$) but formally significant correlation in the tables and text probably are due to small systematic biases in the Cy5/Cy3 ratios that violate the assumption of independent measurement errors used to generate the 95% confidence limits. Therefore, these small correlation values should be treated as not significant. A likely source of uncorrected systematic bias is the partially corrected scanner detector nonlinearity that differently affects the Cy3 and Cy5 detection channels.

The 1 μg/ml FK506 treatment signature was compared with more than 40 unrelated deletion mutant strain or drug signatures. These control profiles had correlation coefficients with the FK506 profile that were distributed around zero (mean $\rho = -0.03$) with a standard deviation of 0.16 (data not shown), and none had correlations greater than $\rho = 0.38$. Similarly, the calcineurin mutant strain signature correlated well with the CsA treatment signature ($\rho = 0.71 \pm 0.04$) but not with the signatures from the negative controls (mean $\rho = -0.02$ with a standard deviation of 0.18).

Quality controls. End-to-end checks on expression ratio measurement accuracy were provided by analyzing the variance in repeated hybridizations using the same mRNA labeled with both Cy3 and Cy5, and also using Cy3 and Cy5 mRNA samples isolated from independent cultures of the same nominal strain and conditions. Biases undetected with this procedure, such as gene-specific biases presumably due to differential incorporation of Cy3- and Cy5-dUTP into cDNA, were minimized by doing hybridizations in fluor-reversed pairs, in which the Cy3/Cy5 labeling of the biological conditions was reversed in one experiment with respect to the other. The expression ratio for each gene is then the ratio of ratios between the two experiments in the pair. Other biases are removed by algorithmic numerical de-trending. The magnitude of these biases in the absence of de-trending and fluor reversal is typically about 30% in the ratio, but may be as high as twofold for some ORFs.

Expression ratios are based on mean intensities over each spot. Some

smaller spots have fewer image pixels in the average. This does not degrade accuracy noticeably until the number of pixels falls below ten, in which case the spot is rejected from the data set. 'Wander' of spot positions with respect to the nominal grid is adaptively tracked in array subregions by the image processing software. Unequal spot 'wander' within a subregion greater than half-a-spot spacing is a difficulty for the automated quantitating algorithms; in this case, the spot is rejected from analysis based on human inspection of the 'wander'. Any spots partially overlapping are excluded from the data set. Less than 1% of spots typically are rejected for these reasons.

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The Transcriptional Program in the Response of Human Fibroblasts to Serum

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The temporal program of gene expression during a model physiological response of human cells, the response of fibroblasts to serum, was explored with a complementary DNA microarray representing about 8600 different human genes. Genes could be clustered into groups on the basis of their temporal patterns of expression in this program. Many features of the transcriptional program appeared to be related to the physiology of wound repair, suggesting that fibroblasts play a larger and richer role in this complex multicellular response than had previously been appreciated.

The response of mammalian fibroblasts to serum has been used as a model for studying growth control and cell cycle progression (1). Normal human fibroblasts require growth factors for proliferation in culture; these growth factors are usually provided by fetal bovine serum (FBS). In the absence of growth factors, fibroblasts enter a nondividing state, termed G_0, characterized by low metabolic activity. Addition of FBS or purified growth factors induces proliferation of the fibroblasts; the changes in gene expression that accompany this proliferative response have been the subject of many studies, and the responses of dozens of genes to serum have been characterized.

We took a fresh look at the response of human fibroblasts to serum, using cDNA microarrays representing about 8600 distinct human genes to observe the temporal program of transcription that underlies this response. Primary cultured fibroblasts from human neonatal foreskin were induced to enter a quiescent state by serum deprivation for 48 hours and then stimulated by addition of medium containing 10% FBS (2). DNA microarray hybridization was used to measure the temporal changes in mRNA levels of 8613 human genes (3) at 12 times, ranging from 15 min to 24 hours after serum stimulation. The cDNA made from purified mRNA from each sample was labeled with the fluorescent dye Cy5 and mixed with a common reference probe consisting of cDNA made from purified mRNA from the quiescent

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Fig. 1. The same section of the microarray is shown for three independent hybridizations comparing RNA isolated at the 8-hour time point after serum treatment to RNA from serum-deprived cells. Each microarray contained 9996 elements, including 9804 human cDNAs, representing 8613 different genes. mRNA from serum-deprived cells was used to prepare cDNA labeled with Cy3–deoxyuridine triphosphate (dUTP), and mRNA harvested from cells at different times after serum stimulation was used to prepare cDNA labeled with Cy5-dUTP. The two cDNA probes were mixed and simultaneously hybridized to the microarray. The image of the subsequent scan shows genes whose mRNAs are more abundant in the serum-deprived fibroblasts (that is, suppressed by serum treatment) as green spots and genes whose mRNAs are more abundant in the serum-treated fibroblasts as red spots. Yellow spots represent genes whose expression does not vary substantially between the two samples. The arrows indicate the spots representing the following genes: 1, protein disulfide isomerase-related protein P5; 2, IL-8 precursor; 3, EST AA057170; and 4, vascular endothelial growth factor.
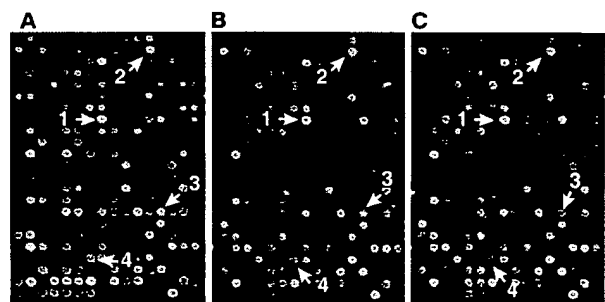
culture (time zero) labeled with a second fluorescent dye, Cy3 (*4*). The color images of the hybridization results (Fig. 1) were made by representing the Cy3 fluorescent image as green and the Cy5 fluorescent image as red and merging the two color images.

Diverse temporal profiles of gene expression could be seen among the 8613 genes surveyed in this experiment (Fig. 2); many of these genes (about half) were unnamed expressed sequence tags (ESTs) (*5*). Although diverse patterns of expression were observed, the orderly choreography of the expression program became apparent when the results were analyzed by a clustering and display method developed in our laboratory for analyzing genome-wide gene expression data (*6*). An example of such an analysis, here applied to a subset of 517 genes whose expression changed substantially in response to serum (*7*), is shown in Fig. 2. The entire detailed data set underlying Fig. 2 is available as a tab-delimited table (in cluster order) at the *Science* Web site (www.sciencemag.org/feature/data/984559.shl). In addition, the entire, larger data set for the complete set of genes analyzed in this experiment can be found at a Web site maintained by our laboratory (genome-www.stanford.edu/serum) (*8*).

One measure of the reliability of the changes we observed is inherent in the expression profiles of the genes. For most genes whose expression levels changed, we could see a gradual change over a few time points, which thus effectively provided independent measurements for almost all of the observations. An additional check was provided by the inclusion of duplicate and, in a few cases, multiple array elements representing the same gene for about 5% of the genes included in this microarray. In addition, three independent hybridizations to different microarrays with mRNA samples from cells harvested 8 hours after serum addition showed good correlation (Fig. 1). As an independent test, we measured the expression levels of several genes using the TaqMan 5′ nuclease fluorigenic quantitative polymerase chain reaction (PCR) assay (*9*). The expression profiles of the genes, as measured by these two independent methods, were very similar (Fig. 3) (*10*).

The transcriptional response of fibroblasts to serum was extremely rapid. The immediate response to serum stimulation was dominated by genes that encode transcription factors and other proteins involved in signal transduction. The mRNAs for several genes [including c-FOS, JUN B, and mitogen-activated protein (MAP) kinase phosphatase-1 (MKP1)] were detectably induced within 15 min after serum stimulation (Fig. 4, A and B). Fifteen of the genes that were observed to be induced by serum encode known or suspected regulators of transcription (Fig. 4B). All but one were immediate-early genes—their induction was not inhibited by cycloheximide (*11*). This class of genes could be distinguished into those whose induction was transient (Fig. 2, cluster E) and those whose mRNA levels remained induced for much longer (Fig. 2, clusters I and J). Some features of the immediate response appeared to be directed at adaptation to the initiating signals. We observed a marked induction of mRNA encoding MKP1, a dual-specificity phosphatase that modulates the activity of the ERK1 and ERK2 MAP kinases (*12*). The coincidence of the peak of expression of genes in cluster E (Fig. 2) with that of MKP1 (Fig. 4A) suggests the possibility

Fig. 2. Cluster image showing the different classes of gene expression profiles. Five hundred seventeen genes whose mRNA levels changed in response to serum stimulation were selected (*7*). This subset of genes was clustered hierarchically into groups on the basis of the similarity of their expression profiles by the procedure of Eisen *et al.* (*6*). The expression pattern of each gene in this set is displayed here as a horizontal strip. For each gene, the ratio of mRNA levels in fibroblasts at the indicated time after serum stimulation ("unsync" denotes exponentially growing cells) to its level in the serum-deprived (time zero) fibroblasts is represented by a color, according to the color scale at the bottom. The graphs show the average expression profiles for the genes in the corresponding "cluster" (indicated by the letters A to J and color coding). In every case examined, when a gene was represented by more than one array element, the multiple representations in this set were seen to have identical or very similar expression profiles, and the profiles corresponding to these independent measurements clustered either adjacent or very close to each other, pointing to the robustness of the clustering algorithm in grouping genes with very similar patterns of expression.
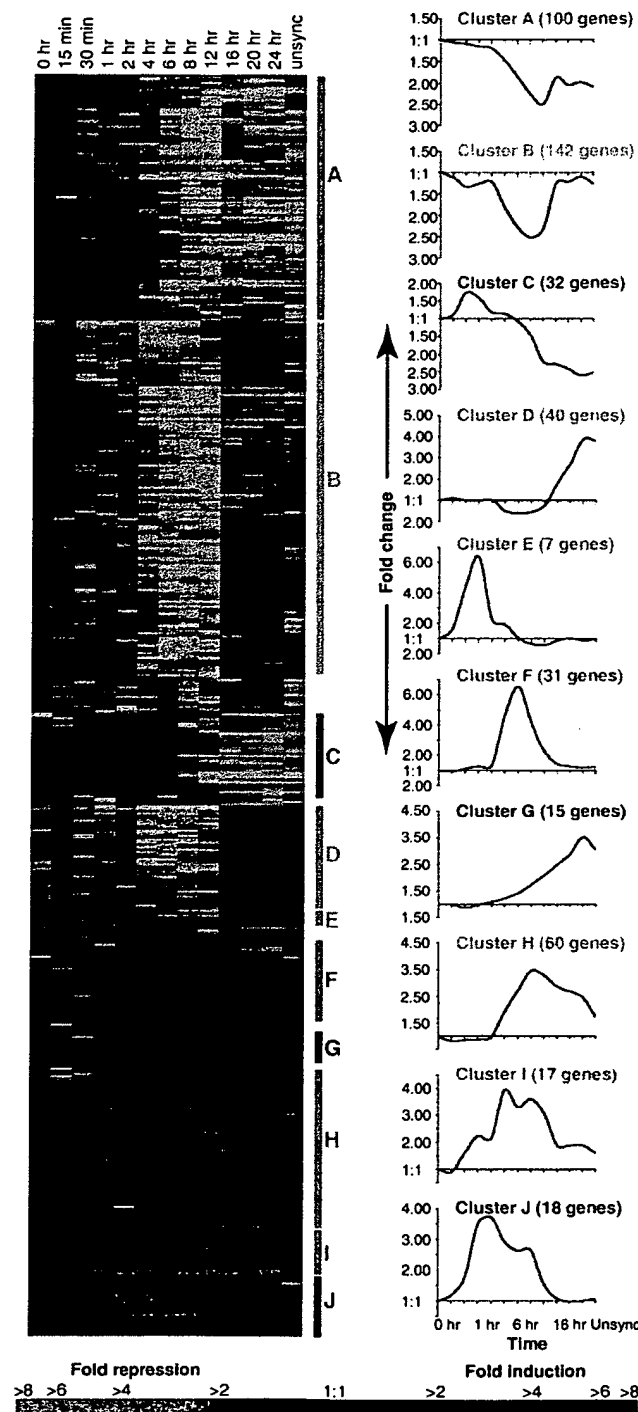
that continued activity of the MAP kinase pathway is required to maintain induction of these genes but not of those with sustained expression (clusters I and J). The gene encoding a second member of the dual-specificity MAP kinase phosphatase family, known as dual-specificity protein phosphatase 6/pyst2, was induced later, at about 4 hours after serum stimulation. Genes encoding diverse other proteins with roles in signal transduction, ranging from cell-surface receptors [for example, the sphingosine 1-phosphate receptor (EDG-1), the vascular endothelial growth factor receptor, and the type II BMP receptor] to regulators of G-protein signaling (for example, NET1/p115 rho GEF) to DNA-binding transcription factors, were induced by serum (Fig. 4A).

The reprogramming of the regulatory circuits in response to serum involved not only induction of transcription factors but also reduced expression of many transcriptional regulators—some of which may play roles in maintaining the cells in G_0 or in priming them to react to wounding (Fig. 4C). Perhaps as a consequence of the historical focus on genes induced by serum stimulation of fibroblasts, the set of transcription factors whose expression diminished upon serum stimulation has been less well characterized.

Genes known or likely to be involved in controlling and mediating the proliferative response showed distinctive patterns of regulation. Several genes whose products inhibit progression of the cell-division cycle, such as p27 Kip1, p57 Kip2, and p18, were expressed in the quiescent fibroblasts and down-regulated before the onset of cell division. The nadir in the mRNA levels for these genes occurred between 6 and 12 hours after serum stimulation (Fig. 5A), coincident with the passage of the fibroblasts through G_1. The levels of the transcript encoding the WEE1-like protein kinase, which is believed to inhibit mitosis by phosphorylation of Cdc2, diminished between 4 and 8 to 12 hours after serum addition (Fig. 5A), well

before the onset of M phase at around 16 hours, raising the possibility of an additional role for Wee1 in an earlier stage of the cell cycle or in regulating the G_0 to G_1 transition. Several genes induced in the first few hours after serum stimulation, such as the helix-loop-helix proteins ID2 and ID3 and EST AA016305, a gene with homology to G_1-S cyclins, are candidates for roles in promoting the exit from G_0.

Genes involved in mediating progression through the cell cycle were characterized by a distinctive pattern of expression (Fig. 2, cluster D), reflecting the coincidence of their expression with the reentry of the stimulated fibroblasts into the cell-division cycle. The stimulated fibroblasts replicated their DNA about 16 hours after serum treatment. This timing was reflected by the induction of mRNA encoding both subunits of ribonucleotide reductase and PCNA, the processivity factor for DNA polymerase epsilon and delta. Cyclin A, Cyclin B1, Cdc2, and CDC28 kinase, regulators of passage through the S phase and the transition from G_2 to M phase, were induced at about 16 to 20 hours after serum addition. The kinase in the Cyclin B1–CDK pair needs to be activated by phosphorylation. The gene encoding Cyclin-dependent kinase 7 (CDK7; a homolog of Xenopus MO15 cdk-activating kinase) was induced in parallel with the Cdc2 and Cdc28 kinases (Fig. 5A), suggesting a potential role for CDK7 in mediating M phase. DNA topoisomerase II α, required for chromosome segregation at mitosis; Mad2, a component of the spindle checkpoint that prevents completion of mitosis (anaphase) if chromosomes are not attached to the spindle; and the kinetochore protein CENP-F all showed a similar expression profile.

In the hours after the serum stimulus, one of the most striking features of the unfolding transcriptional program was the appearance of numerous genes with known roles in processes relevant to the physiology of wound healing.

These included both genes involved in the direct role played by fibroblasts in remodeling of the clot and the extracellular matrix and, more notably, genes encoding proteins involved in intercellular signaling (Fig. 5). Genes induced in this program encode products that can (i) participate in the dynamic process of clotting, clot dissolution, and remodeling and perhaps contribute to hemostasis by promoting local vasoconstriction (for example, endothelin-1); (ii) promote chemotaxis and activation of neutrophils (for example, COX2) and recruitment and extravasation of monocytes and macrophages (for example, MCP1); (iii) promote chemotaxis and activation of T lymphocytes [for example, interleukin-8 (IL-8)] and B lymphocytes (for example, ICAM-1), thus providing both innate and antigen-specific defenses against wound infection and recruiting the phagocytic cells that will be required to clear out the debris during remodeling of the wound; (iv) promote angiogenesis and neovascularization (for example, VEGF) through newly forming tissue; (v) promote migration and proliferation of fibroblasts (for example, CTGF) and their differentiation into myofibroblasts (for example, Vimentin); and (vi) promote migration and proliferation of keratinocytes, leading to reepithelialization of the wound (for example, FGF7), and promote proliferation of melanocytes, perhaps contributing to wound hyperpigmentation (for example, FGF2).

Coordinated regulation of groups of genes whose products act at different steps in a common process was a recurring theme. For example, Furin, a prohormone-processing protease required for one of the processing steps in the generation of active endothelin, was induced in parallel with induction of the gene encoding the precursor of endothelin-1 (Fig. 5E) (13). Conversely, expression of CALLA/CD10, a membrane metalloprotease that degrades endothelin-1 and other peptide mediators of acute inflammation, was re-
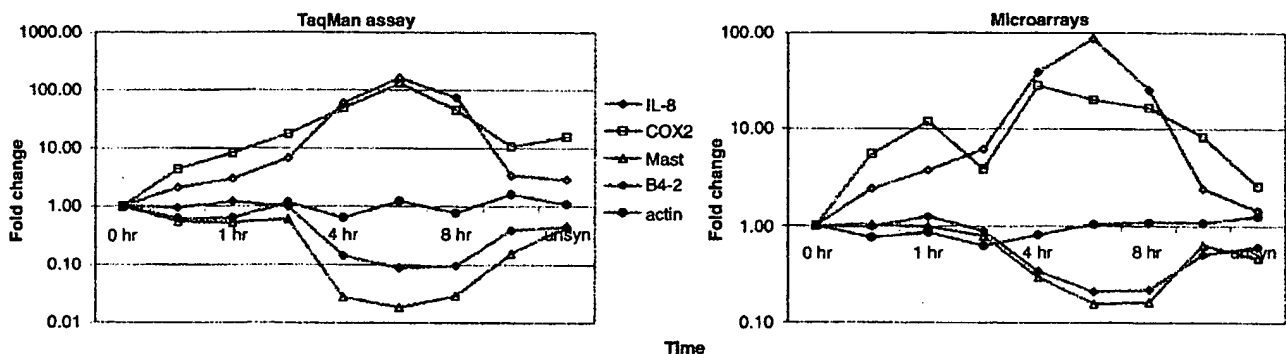
Fig. 3. Independent verification of microarray quantitation. Relative mRNA levels of the indicated genes (Mast, mast/stem cell growth factor receptor) were measured with the TaqMan 5' nuclease fluorigenic quantitative PCR assay (9) (left) in the same samples that were used to prepare probes for microarray hybridizations (right). Data from the TaqMan analysis were normalized to mRNA concentrations and plotted relative to the level at time zero, so that the results could be compared with those from the microarray hybridizations. In general, quantitation with the two methods gave very similar results (10).

duced. A second example is provided by a set of five genes involved in the biosynthesis of cholesterol (Fig. 5I). The mRNAs encoding each of these enzymes showed sharply diminished expression beginning 4 to 6 hours after serum stimulation of fibroblasts. A likely explanation for the coordinated down-regulation of the cholesterol biosynthetic pathway is that serum provides cholesterol to fibroblasts through low-density lipoproteins, whereas in the absence of the cholesterol provided by serum, endogenous cholesterol biosynthesis in fibroblasts is required.

Many of the previously studied genes that we observed to be regulated in this program have no recognized role in any aspect of wound healing or fibroblast proliferation. Their identification in this study may therefore point to previously unknown aspects of these processes. A few selected genes in this group are shown in Fig. 5H. The stanniocalcin gene, for example (Fig. 5H), encodes a secreted protein without a clearly identified function in human cells (14, 15). Its induction in serum-stimulated fibro-

blasts suggests the possibility that it may play a role in the wound-healing process, perhaps serving as a signal in mediating inflammation or angiogenesis.

One of the most important results of this exploration was the discovery of over 200 previously unknown genes whose expression was regulated in specific temporal patterns during the response of fibroblasts to serum. For example, 13 of the 40 genes in cluster D (Fig. 2) have descriptive names that reflect their putative function. Nine of these 13 genes (69%) encode proteins that play roles in cell cycle progression, particularly in DNA replication and the G_2-M transition. This enrichment for cell cycle–related genes suggests that some of the

unnamed genes in this cluster—for example, EST W79311 and EST R13146, neither of which have sequence similarity to previously characterized genes—may represent previously unknown genes involved in this part of the cell cycle. Similarly, a remarkable fraction of genes that were grouped into cluster F on the basis of their expression profiles encoded proteins involved in intercellular signaling (Fig. 2), suggesting that a similar role should be considered for the many unnamed genes in this cluster. A disproportionately large fraction of the genes whose transcription diminished upon serum stimulation were unnamed ESTs.

Our intention was to use this experiment as a model to study the control of the transition

Fig. 4. "Reprogramming" of fibroblasts. Expression profiles of genes whose function is likely to play a role in the reprogramming phase of the response are shown with the same representation as in Fig. 2. In the cases in which a gene was represented by more than one element in the microarray, all measurements are shown. The genes were grouped into categories on the basis of our knowledge of their most likely role. Some genes with pleiotropic roles were included in more than one category.

Fig. 5. The transcriptional response to serum suggests a multifaceted role for fibroblasts in the physiology of wound healing. The features of the transcriptional program of fibroblasts in response to serum stimulation that appear to be related to various aspects of the wound-healing process and fibroblast proliferation are shown with the same convention for representing changes in transcript levels as was used in Figs. 2 and 4. (A) Cell cycle and proliferation, (B) coagulation and hemostasis, (C) inflammation, (D) angiogenesis, (E) tissue remodeling, (F) cytoskeletal reorganization, (G) reepithelialization, (H) unidentified role in wound healing, and (I) cholesterol biosynthesis. The numbers in (C) and (G) refer to genes whose products serve as signals to neutrophils (C1), monocytes and macrophages (C2), T lymphocytes (C3), B lymphocytes (C4), and melanocytes (G1).

from G_0 to a proliferating state. However, one of the defining characteristics of genome-scale expression profiling experiments is that the examination of so many diverse genes opens a window on all the processes that actually occur and not merely the single process one intended to observe. Serum, the soluble fraction of clotted blood, is normally encountered by cells in vivo in the context of a wound. Indeed, the expression program that we observed in response to serum suggests that fibroblasts are programmed to interpret the abrupt exposure to serum not as a general mitogenic stimulus but as a specific physiological signal, signifying a wound. The proliferative response that we originally intended to study appeared to be part of a larger physiological response of fibroblasts to a wound. Other features of the transcriptional response to serum suggest that the fibroblast is an active participant in a conversation among the diverse cells that work together in wound repair, interpreting, amplifying, modifying, and broadcasting signals controlling inflammation, angiogenesis, and epithelial regrowth during the response to an injury.

We recognize that these in vitro results almost certainly represent a distorted and incomplete rendering of the normal physiological response of a fibroblast to a wound. Moreover, only the responses elicited directly by exposure of fibroblasts to serum were examined. The subsequent signals from other cellular participants in the normal wound-healing process would certainly provoke further evolution of the transcriptional program in fibroblasts at the site of a wound, which this experiment cannot reveal. Nevertheless, we believe that the picture that emerged strongly suggests a much larger and richer role for the fibroblast in the orchestration of this important physiological process than had previously been suspected.

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3. The National Center for Biotechnology Information maintains the UniGene database as a resource for partitioning human sequences contained in GenBank into clusters representing distinct transcripts or genes (18, 19). At the time this work began, this database contained about 40,000 such clusters. We selected a subset of 10,000 of these UniGene clusters for inclusion on gene expression microarrays. UniGene clusters were included only if they contained at least one clone from the I.M.A.G.E. human cDNA collection (20), so that a physical clone could easily be obtained (all I.M.A.G.E. clones are available commercially from a number of vendors). We attempted to include as complete as possible a set of the "named" human genes (about 4000) and genes that appeared to be closely related to named genes in other organisms (about an additional 2000). The remaining 4000 clones were chosen from among the "anonymous" UniGene clusters on the basis of inclusion on the human transcript map (www.ncbi.nlm.nih.gov/SCIENCE96/) and the lack of apparent homology to any other genes in the selected set. A physical clone representing each of the selected genes was obtained from Research Genetics. This "10K set" is included in a more recent "15K set" described at www.nhgri.nih.gov/DIR/LCG/15K/HTML/p15Ktop.html. Of these clones, 472 are absent from the current edition of UniGene and were presumed to be distinct genes. The remainders represent 8141 distinct clusters, or human genes, in UniGene. These clones, thus presumed to represent 8613 different genes, were used to print microarrays according to methods described previously (21, 22).
4. One microgram of mRNA was used for making fluorescently labeled cDNA probes for hybridizing to the microarrays, with the protocol described previously (23). mRNA from the large batch of serum-starved cells was used to make cDNA labeled with Cy3. The Cy3-labeled cDNA from this batch of serum-starved cells served as the common reference probe in all hybridizations. mRNA samples from cells harvested immediately before serum stimulation, at intervals after serum stimulation, and from exponentially growing cells were used to make cDNA labeled with Cy5. Ten micrograms of yeast tRNA, 10 μg of polydeoxyadenylic acid, and 20 μg of human CoT1 DNA (Gibco-BRL) were added to the mixture of labeled probes in a solution containing 3× standard saline citrate (SSC) and 0.3% SDS and allowed to prehybridize at room temperature for 30 min before the probe was added to the surface of the microarray. Hybridizations, washes, and fluorescent scans were performed as described previously (23, 24). All measurements, totaling more than 180,000 differential expression measurements, were stored in a computer database for analysis and interpretation.
5. The nominal identities of a number of cDNAs (currently about 3750) on the microarray were verified by sequencing. The clones that were sequenced included many of the genes whose expression changed substantially upon serum stimulation, as well as a large number of genes whose expression did not change substantially in the course of this experiment. About 85% of the clones on the current version of this microarray that were checked by resequencing were correctly identified. In all the figures, gene names or EST numbers are given only for those genes on the microarrays whose identities were reconfirmed by resequencing. In the cases where a human gene has more than one name in the literature, we have tried to use the name that is most evocative of its presumed role in this context. The remainder of the clones have been assigned a temporary identification number (format: SID#######) and a putative identity pending sequence verification. The correct identities of these genes will be posted at our Web site (genome-www.stanford.edu/serum) as they are confirmed by resequencing.
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article

Systematic variation in gene expression patterns in human cancer cell lines

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We used cDNA microarrays to explore the variation in expression of approximately 8,000 unique genes among the 60 cell lines used in the National Cancer Institute's screen for anti-cancer drugs. Classification of the cell lines based solely on the observed patterns of gene expression revealed a correspondence to the ostensible origins of the tumours from which the cell lines were derived. The consistent relationship between the gene expression patterns and the tissue of origin allowed us to recognize outliers whose previous classification appeared incorrect. Specific features of the gene expression patterns appeared to be related to physiological properties of the cell lines, such as their doubling time in culture, drug metabolism or the interferon response. Comparison of gene expression patterns in the cell lines to those observed in normal breast tissue or in breast tumour specimens revealed features of the expression patterns in the tumours that had recognizable counterparts in specific cell lines, reflecting the tumour, stromal and inflammatory components of the tumour tissue. These results provided a novel molecular characterization of this important group of human cell lines and their relationships to tumours *in vivo*.

Introduction

Cell lines derived from human tumours have been extensively used as experimental models of neoplastic disease. Although such cell lines differ from both normal and cancerous tissue, the inaccessibility of human tumours and normal tissue makes it likely that such cell lines will continue to be used as experimental models for the foreseeable future. The National Cancer Institute's Developmental Therapeutics Program (DTP) has carried out intensive studies of 60 cancer cell lines (the NCI60) derived from tumours from a variety of tissues and organs[1–4]. The DTP has assessed many molecular features of the cells related to cancer and chemotherapeutic sensitivity, and has measured the sensitivities of these 60 cell lines to more than 70,000 different chemical compounds, including all common chemotherapeutics (http://dtp.nci.nih.gov). A previous analysis of these data revealed a connection between the pattern of activity of a drug and its method of action. In particular, there was a tendency for groups of drugs with similar patterns of activity to have related methods of action[3,5–7].

We used DNA microarrays to survey the variation in abundance of approximately 8,000 distinct human transcripts in these 60 cell lines. Because of the logical connection between the function of a gene and its pattern of expression, the correlation of gene expression patterns with the variation in the phenotype of the cell can begin the process by which the function of a gene can be inferred. Similarly, the patterns of expression of known genes can

reveal novel phenotypic aspects of the cells and tissues studied[8–10]. Here we present an analysis of the observed patterns of gene expression and their relationship to phenotypic properties of the 60 cell lines. The accompanying report[11] explores the relationship between the gene expression patterns and the drug sensitivity profiles measured by the DTP. The assessment of gene expression patterns in a multitude of cell and tissue types, such as the diverse set of cell lines we studied here, under diverse conditions *in vitro* and *in vivo*, should lead to increasingly detailed maps of the human gene expression program and provide clues as to the physiological roles of uncharacterized genes[11–16]. The databases, plus tools for analysis and visualization of the data, are available (http://genome-www.stanford.edu/nci60 and http://discover.nci.nih.gov).

Results

We studied gene expression in the 60 cell lines using DNA microarrays prepared by robotically spotting 9,703 human cDNAs on glass microscope slides[17,18]. The cDNAs included approximately 8,000 different genes: approximately 3,700 represented previously characterized human proteins, an additional 1,900 had homologues in other organisms and the remaining 2,400 were identified only by ESTs. Due to ambiguity of the identity of the cDNA clones used in these studies, we estimated that approximately 80% of the genes in these experiments were correctly identified. The identities of approximately 3,000 cDNAs

Fig. 1 Gene expression patterns related to the tissue of origin of the cell lines. Two-dimensional hierarchical clustering was applied to expression data from a set of 1,161 cDNAs measured across 64 cell lines. The 1,161 cDNAs were those (of 9,703 total) with transcript levels that varied by at least sevenfold ($\log_2$ (ratio) >2.8) relative to the reference pool in at least 4 of 60 cell lines. This effectively selected genes with the greatest variation in expression level across the 60 cell lines (including those genes not well represented in the reference pool), and therefore highlighted those gene expression patterns that best distinguished the cell lines from one another. Data from 64 hybridizations were used, one for each cell line plus the two additional independent representations of each of the cell lines K562 and MCF7. The two cell lines represented in triplicate were correspondingly weighted for the gene clustering so that each of the 60 cell lines contributed equally to the clustering. *a*, The cell-line dendrogram, with the terminal branches coloured to reflect the ostensible tissue of origin of the cell line (red, leukaemia; green, colon; pink, breast; purple, prostate; light blue, lung; orange, ovarian; yellow, renal; grey, CNS; brown, melanoma; black, unknown (NCI/ADR-RES)). The scale to the right of the dendrogram depicts the correlation coefficient represented by the length of the dendrogram branches connecting pairs of nodes. Note that the two triplets of replicated cell lines (K562 and MCF7) cluster tightly together and were well differentiated from even the most closely related cell lines, indicating that this clustering of cell lines is based on characteristic variations in their gene expression patterns rather than artefacts of the experimental procedures. *b*, A coloured representation of the data table, with the rows (genes) and columns (cell lines) in cluster order. The dendrogram representing hierarchical relationships between genes was omitted for clarity, but is available (http://genome-www.stanford.edu/nci60). The colour in each cell of this table reflects the mean-adjusted expression level of the gene (row) and cell line (column). The colour scale used to represent the expression ratios is shown. The labels '3a–3d' in (*b*) refer to the clusters of genes shown in detail in Fig. 3.

from these experiments have been sequence-verified, including all of those referred to here by name.

Each hybridization compared Cy5-labelled cDNA reverse transcribed from mRNA isolated from one of the cell lines with Cy3-labelled cDNA reverse transcribed from a reference mRNA sample. This reference sample, used in all hybridizations, was prepared by combining an equal mixture of mRNA from 12 of the cell lines (chosen to maximize diversity in gene expression as determined primarily from two-dimensional gel studies[2]). By comparing cDNA from each cell line with a common reference, variation in gene expression across the 60 cell lines could be inferred from the observed variation in the normalized Cy5/Cy3 ratios across the hybridizations.

To assess the contribution of artefactual sources of variation in the experimentally measured expression patterns, K562 and MCF7 cell lines were each grown in three independent cultures, and the entire process was carried out independently on mRNA extracted from each culture. The variance in the triplicate fluorescence ratio measurements approached a minimum when the fluorescence signal was greater than approximately 0.4% of the measurable total signal dynamic range above background in either channel of the hybridization. We selected the subset of spots for which significant signal was present in both the numerator and denominator of the ratios by this criterion to identify the best-measured spots. The pair-wise correlation coefficients for the triplicates of the set of genes that passed this quality control level (6,992 spots included for the MCF7 samples and 6,161 spots for K562) ranged from 0.83 to 0.92 (for graphs and details, see http://genome-www.stanford.edu/nci60).

To make the orderly features in the data more apparent, we used a hierarchical clustering algorithm[19,20] and a pseudo-colour visu-

alization matrix[3,21]. The object of the clustering was to group cell lines with similar repertoires of expressed genes and to group genes whose expression level varied among the 60 cell lines in a similar manner. Clustering was performed twice using different subsets of genes to assess the robustness of the analysis. In one case (Fig. 1), we concentrated on those genes that showed the most variation in expression among the 60 cell lines (1,167 total). A second analysis (Fig. 2) included all spots that were thought to be well measured in the reference set (6,831 spots).

Gene expression patterns related to the histologic origins of the cell lines

The most notable property of the clustered data was that cell lines with common presumptive tissues of origin grouped together (Figs 1*a* and 2). Cell lines derived from leukaemia, melanoma, central nervous system, colon, renal and ovarian tissue were clustered into independent terminal branches specific to their respective organ types with few exceptions. Cell lines derived from non-small lung carcinoma and breast tumours were distributed in multiple different terminal branches suggesting that their gene expression patterns were more heterogeneous.

Many of these coherent cell line clusters were distinguished by the specific expression of characteristic groups of genes (Fig. 3*a–d*). For example, a cluster of approximately 90 genes was highly expressed in the melanoma-derived lines (Fig. 3*c*). This set was enriched for genes with known roles in melanocyte biology, including tyrosinase and dopachrome tautomerase (TYR and DCT; two subunits of an enzyme complex involved in melanin synthesis[22]), MART1 (MLANA; which is being investigated as a target for immunotherapy of melanoma[23]) and S100-β (S100B; which has been used as an antigenic marker in the diagnosis of

Fig. 2 Gene expression patterns related to other cell-line phenotypes. *a*, We applied two-dimensional hierarchical clustering to expression data from a set of 6,831 cDNAs measured across the 64 cell lines. The 6,831 cDNAs were those with a minimum fluorescence signal intensity of approximately 0.4% of the dynamic range above background in the reference channel in each of the six hybridizations used to establish reproducibility. This effectively selected those spots that provided the most reliable ratio measurements and therefore identified a subset of genes useful for exploring patterns comprised of those whose variation in expression across the 60 cell lines was of moderate magnitude. *b*, Cluster-ordered data table. *c*, Doubling time of cell lines. Cell lines are given in cluster order. Values are plotted relative to the mean. Doubling times greater than the mean are shown in green, those with doubling time less than the mean are shown in red. *d*, Three related gene clusters that were enriched for genes whose expression level variation was correlated with cell line proliferation rate. Each of the three gene clusters (clustered solely on the basis of their expression patterns) showed enrichment for sets of genes involved in distinct functional categories (for example, ribosomal genes versus genes involved in pre-RNA splicing). *e*, Gene cluster in which all characterized and sequence-verified cDNAs encode genes known to be regulated by interferons. *f*, Gene cluster enriched for genes that have been implicated in drug metabolism (indicated by asterisks). A further property of the gene clustering evident here and in Fig. 2 is the strong tendency for redundant representations of the same gene to cluster immediately adjacent to one another, even within larger groups of genes with very similar expression patterns. In addition to illustrating the reproducibility and consistency of the measurements, and providing independent confirmation of many of our measurements, this property also demonstrates that these, and probably all, genes have nearly unique patterns of variation across the 60 cell lines. If this were not the case, and multiple genes had identical patterns of variation, we would not expect to be able to distinguish, by clustering on the basis of expression variation, duplicate copies of individual genes from the other genes with identical expression patterns.

d proliferation cluster

e interferon cluster

f drug metabolism cluster
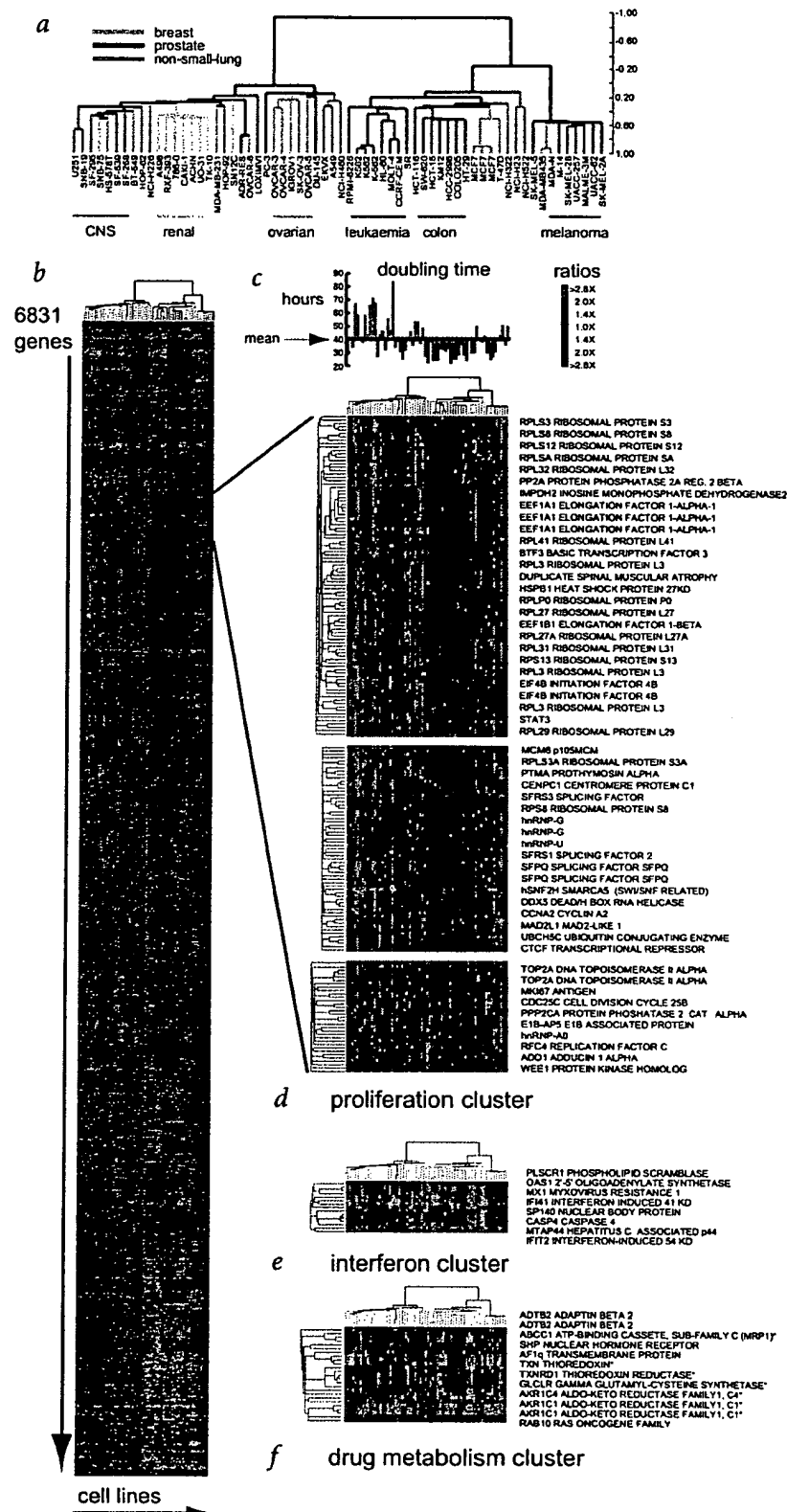
melanoma). LOXIMVI, the seventh line designated as melanoma in the NCI60, did not show this characteristic pattern. Although isolated from a patient with melanoma, LOXIMVI has previously been noted to lack melanin and other markers useful for identification of melanoma cells[1].

Paradoxically, two related cell lines (MDA-MB435 and MDA-N), which were derived from a single patient with breast cancer and have been conventionally regarded as breast cancer cell lines, shared expression of the genes associated with melanoma. MDA-MB435 was isolated from a pleural effusion in a patient with metastatic ductal adenocarcinoma of the breast[24,25]. It remains possible that the origin of the cell line was a breast cancer, and that its gene expression pattern is related to the neuroendocrine features of some breast cancers[26]. But our results suggest that this cell line may have originated from a melanoma, raising the possibility that the patient had a co-existing occult melanoma.

The higher-level organization of the cell-line tree—in which groups span cell lines from different tissue types—also reflected shared biological properties of the tissues from which the cell lines were derived. The carcinoma-derived cell lines were divided into major branches that separated those that expressed genes characteristic of epithelial cells from those that expressed genes more typical of stromal cells. A cluster of genes is shown (Fig. 3b) that is most strongly expressed in cell lines derived from colon carcinomas, six of seven ovarian-derived cell lines and the two breast cancer lines positive for the oestrogen receptor. The named genes in this cluster have been implicated in several aspects of epithelial cell biology[27]. The cluster was enriched for genes whose products are known to localize to the basolateral membrane of epithelial cells, including those encoding components of adherens complexes (for example, desmoplakin (DSP), periplakin (PPL) and plakoglobin (JUP)), an epithelial-expressed cell-cell adhesion molecule (M4S1) and a sodium/hydrogen ion exchanger[28–31] (SLC9A1). It also contained genes that encode putative transcriptional regulators of epithelial morphogenesis, a human homologue of a *Drosophila melanogaster* epithelial-expressed tumour suppressor (LLGL1) and a homeobox gene thought to control calcium-mediated adherence in epithelial cells[32,33] (MSX2).

In contrast, a separate, major branch of the cell-line dendrogram (Fig. 1a) included all glioblastoma-derived cell lines, all renal-cell-carcinoma–derived cell lines and the remaining carcinoma-derived lines. The characteristic set of genes expressed in this cluster included many whose products are involved in stromal cell functions (Fig. 3d). Indeed, the two cell lines originally described as 'sarcoma-like' in appearance (Hs578T, breast carcinosarcoma, and SF539, gliosarcoma) expressed most of these genes[34,35]. Although no single gene was uniformly characteristic of this cluster, each cell line showed a distinctive pattern of expression of genes encoding proteins with roles in synthesis or modification of the extracellular matrix (for example, caldesmon (CALD1), cathepsins, thrombospondin (THBS), lysyl oxidase (LOX) and collagen subtypes). Although the ovarian and most non-small-cell-lung–derived carcinomas expressed genes characteristic of both epithelial cells and stromal cells, they probably clustered with the CNS and renal cell carcinomas in this analysis because genes characteristically expressed in stromal cells were more abundantly represented in this gene set.

Physiological variation reflected in gene expression patterns

A cluster diagram of 6,831 genes (Fig. 2) is useful for exploring clusters of genes whose variation in mRNA levels was not obviously attributable to cell or tissue type. We identified some gene clusters that were enriched for genes involved in specific cellular

processes; the variation in their expression levels may reflect corresponding differences in activity of these processes in the cell lines. For example, a cluster of 1,159 genes (Fig. 2a) included many whose products are necessary for progression through the cell cycle (such as CCNA1, MCM106 and MAD2L1), RNA processing and translation machinery (such as RNA helicases, hnRNPs and translation elongation factors) and traditional pathologic markers used to identify proliferating cells (MKI67). Within this large cluster were smaller clusters enriched for genes with more specialized roles. One cluster was highly enriched for numerous ribosomal genes, whereas another was more enriched for genes encoding RNA-splicing factors. The variation in expression of these ribosomal genes was significantly correlated with variation in the cell doubling time (correlation coefficient of 0.54), supporting the notion that the genes in this cluster were regulated in relation to cell proliferation rate or growth rate in these cell lines.

In a smaller gene cluster (Fig. 2d), all of the named genes were previously known to be regulated by interferons[13,36]. Additional groups of interferon-regulated genes showed distinct patterns of expression (data not shown), suggesting that the NCI60 cell lines exhibited variation in activity of interferon-response pathways, which was reflected in gene expression patterns[36].

Another cluster (Fig. 2e) contained several genes encoding proteins with possible interrelated roles in drug metabolism, including glutamate-cysteine ligase (GLCLC, the enzyme responsible for the rate limiting step of glutathione synthesis), thioredoxin (TXN) and thioredoxin reductase (TXNRD1; enzymes involved in regulating redox state in cells), and MRP1 (a drug transporter known to efficiently transport glutathione-conjugated compounds[37]). The elevated expression of this set of genes in a subset of these cell lines may reflect selection for resistance to chemotherapeutics.

Cell lines facilitate interpretation of gene expression patterns in complex clinical samples

Like many other types of cancer, tumours of the breast typically have a complex histological organization, with connective tissue and leukocytic infiltrates interwoven with tumour cells. To explore the possibility that variation in gene expression in the tumour cell lines might provide a framework for interpreting the expression patterns in tumour specimens, we compared RNA isolated from two breast cancer biopsy samples, a sample of normal breast tissue and the NCI60 cell lines derived from breast cancers (excluding MDA-MB-435 and MDA-N) and leukaemias (Fig. 4). This clustering highlighted features of the gene expression pattern shared between the cancer specimens and individual cell lines derived from breast cancers and leukaemias.

The genes encoding keratin 8 (KRT8) and keratin 19 (KRT19), as well as most of the other 'epithelial' genes defined in the complete NCI60 cell line cluster, were expressed in both of the biopsy samples and the two breast-derived cell lines, MCF-7 and T47D, expressing the oestrogen receptor, suggesting that these transcripts originated in tumour cells with features similar to those of luminal epithelial cells (Fig. 5a). Expression of a set of genes characteristic of stromal cells, including collagen genes (COL3A1, COL5A1 and COL6A1) and smooth muscle cell markers (TAGLN), was a feature shared by the tumour sample and the stromal-like cell lines Hs578T and BT549 (Fig. 5b). This feature of the expression pattern seen in the tumour samples is likely to be due to the stromal component of the tumour. The tumours also shared expression of a set of genes (Fig. 5c) with the multiple myeloma cell line (RPMI-8226), notably including immunoglobulin genes, consistent with the presence of B cells in the tumour (this was confirmed by staining with anti-

a

leukaemia cluster (6 ESTs)

LGT4 LONG QT SYNDROME
HBA1 HEMOGLOBIN ALPHA 1
HBG2 HEMOGLOBIN GAMMA-G
HBG2 HEMOGLOBIN GAMMA-G
GYPA GLYCOPHORIN A
HBA1 HEMOGLOBIN ALPHA 1
H2FAL HISTONE 2A L
HBB HEMOGLOBIN BETA

SLC4A1 SOLUTE CARRIER FAMILY4
WT1 WILMS TUMOR 1
WT1 WILMS TUMOR 1
PECAM1
FLI1 FRIEND LEUKEMIA VIRUS INTEGRATION 1
CLK ASSOCIATED RS CYCLOPHILIN
CD3G
PRKCQ PROTEIN KINASE C THETA
SLA SRC LIKE ADAPTER
CD63
PTPRC PROTEIN TYR PHOSPHATASE RECEPTOR C
PTPRC PROTEIN TYR PHOSPHATASE RECEPTOR C
LEUKOCYTE SPECIFIC TRANSCRIPT 1
CD1C
EV12B
LRMP
ARHGDIB RHO GDP DISSOCIATION INHIBITOR
LCP1 L-PLASTIN
ALOX5AP 5-LIPOXYGENASE ACTIVATING PROTEIN
MPO MYELOPEROXIDASE
CD33
ETS2 V-ETS E26 HOMOLOG 2
GNA15 GUANINE NUCLEOTIDE BINDING A15
PRKCB1 PROTEIN KINASE C BETA 1

b

epithelial cluster (15 ESTs)

ITGB4 INTEGRIN BETA-4
ITGB4 INTEGRIN BETA-4
DLG3 NEUROENDOCRINE-DLG
LGL2 HUMAN GIANT LARVAE HOMOLOG
CDS1 DIACYLGLYCEROL SYNTHASE
PPL PERIPLAKIN
SLC5A1 SOLUTE CARRIER FAMILY 9
ELF3 EPITHELIAL TRANSCRIPTION FACTOR
M451 GA733-2 (Ep-CAM)
AMBP BIKUNIN
MAP7 E-MAP-115
MAP7 E-MAP-115
DSP DESMOPLAKIN
DSP DESMOPLAKIN
DSP DESMOPLAKIN
JUP PLAKOGLOBIN
CLDN4 CLAUDIN 4
LYZ LYSOZYME
GPX2 GLUTATHIONE PEROXIDASE 2
TM4SF3 TUMOR-ASSOCIATED ANTIGEN CO-029
DDC DOPA DECARBOXYLASE
LAMA3 LAMININ ALPHA 3
KRT8 KERATIN 8
KRT7 KERATIN 7
ANXA3 ANNEXIN III
NUCB2 DNA-BINDING PROTEIN NEFA
NUCB2 DNA-BINDING PROTEIN NEFA
TPD52L1 TUMOR PROTEIN D52-LIKE 1
HE4 EXTRACELLULAR PROTEINASE INHIBITOR
MSX2 MSH HOMEOBOX HOMOLOG
PLML PHOSPHOLEMMAN-LIKE

c

melanoma cluster (16 ESTs)

CMOAT
CMOAT
CMOAT
G2 PROTEIN
PTPRZ1 TYROSINE PHOSPHATASE ZETA
MBP MYELIN BASIC PROTEIN
MBP MYELIN BASIC PROTEIN
BCHE BUTYRYLCHOLINESTERASE
SCRAPIE RESPONSIVE PROTEIN 1
ART3 MONO-ADP-RIBOSYLTRANSFERASE
SIAT8 ALPHA2,3-SIALYLTRANSFERASE
ACP5 ACID PHOSPHATASE TYPE 5
RXRG RETINOID X RECEPTOR GAMMA
DCT DOPACHROME TAUTOMERASE
TYR TYROSINASE
MUTYH MUTY HOMOLOG
S100B S-100 BETA
EDNRB ENDOTHELIN RECEPTOR TYPE B
TYRP1 TYROSINASE-RELATED PROTEIN 1
SIAT8A ALPHA2,8-SIALYLTRANSFERASE
MLANA MELANOMA ANTIGEN RECOGNIZED BY T-CELLS
GM48
PDNP2 NUCLEOTIDE PYROPHOSPHATASE 2
ERDA3 CAGH3
ERDA3 CAGH3
CPB2 PLASMA CARBOXYPEPTIDASE B
LGALS3BP GALECTIN-6 BINDING PROTEIN
A2M ALPHA-2-MACROGLOBULIN
A2M ALPHA-2-MACROGLOBULIN
A2M ALPHA-2-MACROGLOBULIN
AACT ALPHA-1-ANTICHYMOTRYPSIN
LAMA4 LAMININ ALPHA 4

d

mesenchymal cluster (67 ESTs)

VACAM1 VASCULAR CELL ADHESION MOLECULE 1
GRO2
TFPI2 TISSUE FACTOR PATHWAY INHIBITOR 2
IL1R1 INTERLEUKIN-1 RECEPTOR TYPE I
SPON1 SPONDIN 1
SPON1 SPONDIN 1
CP CERULOPLASMIN
TNFAIP2 TNF ALPHA-INDUCED PROTEIN 2
MNDA MYELOID CELL NUCLEAR DIFFERENTIATION ANTIGEN
HSD3B1 HYDROXY-DELTA-5-STEROID DEHYDROGENASE
HSD3B1 HYDROXY-DELTA-5-STEROID DEHYDROGENASE
SLPI ANTILEUKOPROTEINASE 1
VTN VITRONECTIN
IF I FACTOR COMPLEMENT
C3 COMPLEMENT
ESM1 ENDOTHELIAL-CELL SPECIFIC 1
IL1B INTERLEUKIN 1 BETA
OPCML OPIOID BINDING PROTEIN
CDH13 H-CADHERIN
CDH13 H-CADHERIN
RARB RETINOIC ACID RECEPTOR BETA
NR3C1 GLUCOCORTICOID RECEPTOR
NTRK3 NEUROTROPHIC TYR KINASE RECEPTOR-RELATED 1
MUC18 MELANOMA ADHESION MOLECULE
MUC18 MELANOMA ADHESION MOLECULE
ANPEP ALANYL AMINOPEPTIDASE
TGM2 TRANSGLUTAMINASE 2
PSG7 PREGNANCY-SPECIFIC BETA 1-GLYCOPROTEIN 7
PSG4 PREGNANCY-SPECIFIC BETA-1 GLYCOPROTEIN 4
F3 THROMBOPLASTIN
F3 THROMBOPLASTIN
SDC1 SYNDECAN 1
ST4-AG TROPHOBLAST GLYCOPROTEIN
EFNB2 EFRIN-B2
LMO7 LIM DOMAIN ONLY 7
EDN1 ENDOTHELIN 1
KRT14 KERATIN 14
TAGLN TRANSGELIN (SM22-ALPHA)
KRT17 KERATIN 17
INHBA INHIBIN BETA A
SPOCK SPARK/OSTEONECTIN
MCP1 MONOCYTE CHEMOTACTIC PROTEIN 1
EFEMP1 EXTRACELLULAR MATRIX PROTEIN 1
EFEMP1 EXTRACELLULAR MATRIX PROTEIN 1
CLU CLUSTERIN
IL6 INTERLEUKIN 6
FN1 FIBRONECTIN 1 (9 replicates)
PLAUR PLASMINOGEN ACTIVATOR UROKINASE RECEPTOR
NRF-2 ETS-RELATED FACTOR
BFGFR BASIC FIBROBLAST GROWTH FACTOR RECEPTOR
HSPB2 HEAT SHOCK 27KD PROTEIN 2
PLCB4 PHOSPHOLIPASE C BETA 4
FGF1 FIBROBLAST GROWTH FACTOR 1
GEM GTPASE
PLOD2 LYSYL HYDROXYLASE 2
CDH2 CADHERIN 2
FHL2 DRAL
CUTL1 CUT DROSOPHILA-LIKE 1
CALD1 CALDESMON
CALD1 CALDESMON
CALD1 CALDESMON
CSF1 COLONY-STIMULATING FACTOR 1
GJA1 CONNEXIN 43
FGF2 FIBROBLAST GROWTH FACTOR 2
FGF2 FIBROBLAST GROWTH FACTOR 2
AXL RECEPTOR
SNK SERUM-INDUCIBLE KINASE
CAV1 CAVEOLIN
CAV1 CAVEOLIN
COL4A1 COLLAGEN TYPE IV ALPHA 1
COL4A1 COLLAGEN TYPE IV ALPHA 1
TPM1 TROPOMYOSIN ALPHA
TPM1 TROPOMYOSIN ALPHA
LOX LYSYL OXIDASE
IGFBP10
CTGF CONNECTIVE TISSUE GROWTH FACTOR
CNN3 CALPONIN 3
THBS1 THROMBOSPONDIN 1
TJP1 TIGHT JUNCTION PROTEIN 1
FAK FOCAL ADHESION KINASE
ANXA2 ANNEXIN II
CAPN2 CALPAIN 2
EPHA2 ECK KINASE
ALCAM ACTIVATED LEUCOCYTE CELL ADHESION MOLECULE
TM4SF1 TUMOR-ASSOCIATED ANTIGEN L6
ITGB5 INTEGRIN BETA-5
ARHE RAS HOMOLOG E
LAMC1 LAMININ GAMMA 1
MIG2 MITOGEN INDUCIBLE GENE 2
SGK
SGK
NEU1 NEURAMINIDASE 1
OAB2
OAB2
DPYSL3 DIHYDROPYRIMIDINASE RELATED PROTEIN 3
DPYSL3 DIHYDROPYRIMIDINASE RELATED PROTEIN 3
PAI1 PLASMINOGEN ACTIVATOR INHIBITOR TYPE I
PAI1 PLASMINOGEN ACTIVATOR INHIBITOR TYPE I
FBN1 FIBRILLIN 1
FBN1 FIBRILLIN 1
THY1
THY1
PDGFRA ALPHA PDGF RECEPTOR
COL4A1 COLLAGEN TYPE IV ALPHA-1
COL4A1 COLLAGEN TYPE IV ALPHA-1
COL5A2 COLLAGEN TYPE V ALPHA 2
CTSL CATHEPSIN L
ANXA8 ANNEXIN VIII
FKHL4 FORKHEAD RELATED 4
CBP2 COLLAGEN BINDING PROTEIN 2
LTBR LYMPHOTOXIN-BETA RECEPTOR
TGFBR2 TRANSFORMING GROWTH FACTOR BETA RECEPTOR II
NRP1 NUCLEAR FACTOR RIP140
CDC2L1 CELL DIVISION CYCLE 2-LIKE 1
EPB4IL2 PROTEIN 4.1-G LIKE 2

Fig. 3 Gene clusters related to tissue characteristics in the cell lines. Enlargements of the regions of the cluster diagram in Fig. 1 showing gene clusters enriched for genes expressed in cell lines of ostensibly similar origins. *a*, Cluster of genes highly expressed in the leukaemia-derived cell lines. Two sub-clusters distinguish genes that were expressed in most leukaemia-derived lines from those expressed exclusively in the eryroblastoid line, K562 (note that the triplicate hybridizations cluster together). *b*, Cluster of genes highly expressed in all colon (7/7) cell lines and all breast-derived cell lines positive for the oestrogen receptor (2/2). This set of genes was also moderately expressed in most ovarian lines (5/6) and some non-small-cell-lung (4/6) lines, but was expressed at a lower level in all renal-cancer–derived lines. *c*, Cluster of genes highly expressed in most melanoma-derived lines (6/7) and two related lines ostensibly derived from breast cancer (MDA-MB435 and MDA-N). *d*, Cluster of genes highly expressed in all glioblastoma (6/6) lines and most lines derived from renal-cell carcinoma (7/8), and more moderately expressed in a subset of carcinoma-derived lines. In all panels, names are shown only for all known genes whose identities were independently re-verified by sequencing. The number of sequence-validated ESTs within the cluster is indicated below the cluster in parentheses. The position of gene names in the adjacent list only approximates their position in the cluster diagram as indicated by the lines connecting the colour chart with the gene list. Complete cluster images with all gene names and accession numbers are available (http://genome-www.stanford.edu/nci60).

Fig. 4 Comparison of the gene expression patterns in clinical breast cancer specimens and cultured breast cancer and leukaemia cell lines. *a*, Two-dimensional hierarchical clustering applied to gene expression data for two breast cancer specimens, a lymph node metastasis from one patient, normal breast and the NCI60 breast and leukaemia-derived cell lines. The gene expression data from tissue specimens was clustered along with expression data from a subset of the NCI60 cell lines to explore whether features of expression patterns observed in specific cell lines could be identified in the tissue samples. Labels indicate gene clusters (shown in detail in Fig. 5) that may be related to specific cellular components of the tumour specimens. *b*, Breast cancer specimen 16 stained with anti-keratin antibodies, showing the complex mix of cell types characteristically found in breast tumours. The arrows highlight the different cellular components of this tissue specimen that were distinguished by the gene expression cluster analysis (Fig. 5).

immunoglobulin antibodies; data not shown). Therefore, distinct sets of genes with co-varying expression among the samples (Fig. 4, arrow) appear to represent distinct cell types that can be distinguished in breast cancer tissue. A fourth cluster of genes, more highly expressed in all of the cell lines than in any of the clinical specimens, was enriched for genes present in the 'proliferation' cluster described above (Fig. 5d). The variation in expression of these genes likely paralleled the difference in proliferation rate between the rapidly cycling cultured cell lines and the much more slowly dividing cells in tissues.

Discussion

Newly available genomics tools allowed us to explore variation in gene expression on a genomic scale in 60 cell lines derived from diverse tumour tissues. We used a simple cluster analysis to identify the prominent features in the gene expression patterns that appeared to reflect 'molecular signatures' of the tissue from which the cells originated. The histological characteristics of the cell lines that dominated the clustering were pervasive enough that similar relationships were revealed when alternative subsets of genes were selected for analysis. Additional features of the expression pattern may be related to variation in physiological attributes such as proliferation rate and activity of interferon-response pathways.

The properties of the tumour-derived cell lines in this study have presumably all been shaped by selection for resistance to host defences and chemotherapeutics and for rapid proliferation in the tissue culture environment of synthetic growth media, fetal bovine serum and a polystyrene substratum. But the primary identifiable factor accounting for variation in gene expression patterns among these 60 cell lines was the identity of the tissue from which each cell line was ostensibly derived. For most of the cell lines we examined, neither physiological nor experimental adaptation for growth in culture was sufficient to overwrite the gene expression programs established during differentiation *in vivo*. Nevertheless, the prominence of mesenchymal features in the cell lines isolated from glioblastomas and carcinomas may reflect a selection for the relative ease of establishment of cell lines expressing stromal characteristics, perhaps combined with physiological adaptation to tissue culture conditions[38–40].

Biological themes linking genes with related expression patterns may be inferred in many cases from the shared attributes of known genes within the clusters. Uncharacterized cDNAs are likely to encode proteins that have roles similar to those of the known gene products with which they appear to be co-regulated. Still, for several clusters of genes, we were unable to discern a common theme linking the identified members of the cluster. Further exploration of their variation in expression under more diverse conditions and more comprehensive investigation of the physiology of the NCI60 cells may provide insight[10]. The relationship of the gene expression patterns to the drug sensitivity patterns measured by the DTP is an example of linking variation in gene expression with more subtle and diverse phenotypic variation[11].

The patterns of gene expression measured in the NCI60 cell lines provide a framework that helps to distinguish the cells that express specific sets of genes in the histologically complex breast cancer specimens[41]. Although it is now feasible to analyse gene expression in micro-dissected tumour specimens[42,43], this observation suggests that it will be possible to explore and interpret some of the biology of clinical tumour samples by sampling them intact. As is useful in conventional morphological pathology, one might be able to observe interactions between a tumour and its microenvironment in this way. These relationships will be clarified by suitable analysis of gene expression patterns from intact as well as dissected tumours[12,14,15,41].

Methods

(100 μl) was purified by gel exclusion, concentrated and resuspended in 3×SSC (10 μl). The PCR products were then printed on treated glass microscope slides using a robot with four printing tips. Detailed protocols for assembling and operating a microarray printer, and printing and experimental application of DNA microarrays are available (http://cmgm.stanford.edu/pbrown).

Preparation of mRNA and reference pool. Cell lines were grown from NCI DTP frozen stocks in RPMI-1640 supplemented with phenol red, glutamine (2 mM) and 5% fetal calf serum. To minimize the contribution of variations in culture conditions or cell density to differential gene expression, we grew each cell line to 80% confluence and isolated mRNA 24 h after transfer to fresh medium. The time between removal from the incubator and lysis of the cells in RNA stabilization buffer was minimized (<1 min). Cells were lysed in buffer containing guanidium isothiocyanate and total RNA was purified with the RNeasy purification kit (Qiagen). We purified mRNA as needed

using a poly(A) purification kit (Oligotex, Qiagen) according to the manufacturer's instructions. Denaturing agarose gel electrophoresis assessed the integrity and relative contamination of mRNA with ribosomal RNA.

The breast tumours were surgically excised from patients and rapidly transported to the pathology laboratory, where samples for microarray analysis were quickly frozen in liquid nitrogen and stored at –80 °C until use. A frozen tumour specimen was removed from the freezer, cut into small pieces (~50–100 mg each), immediately placed into 10–12 ml of Trizol reagent (Gibco-BRL) and homogenized using a PowerGen 125 Tissue Homogenizer (Fisher Scientific), starting at 5,000 r.p.m. and gradually increasing to ~20,000 r.p.m. over a period of 30–60 s. We processed the Trizol/tumour homogenate as described in the Trizol protocol, including an initial step to remove fat. Once total RNA was obtained, we isolated mRNA with a FastTrack 2.0 kit (Invitrogen) using the manufacturer's protocol for isolating mRNA starting from total RNA. The normal breast samples were obtained from Clontech.
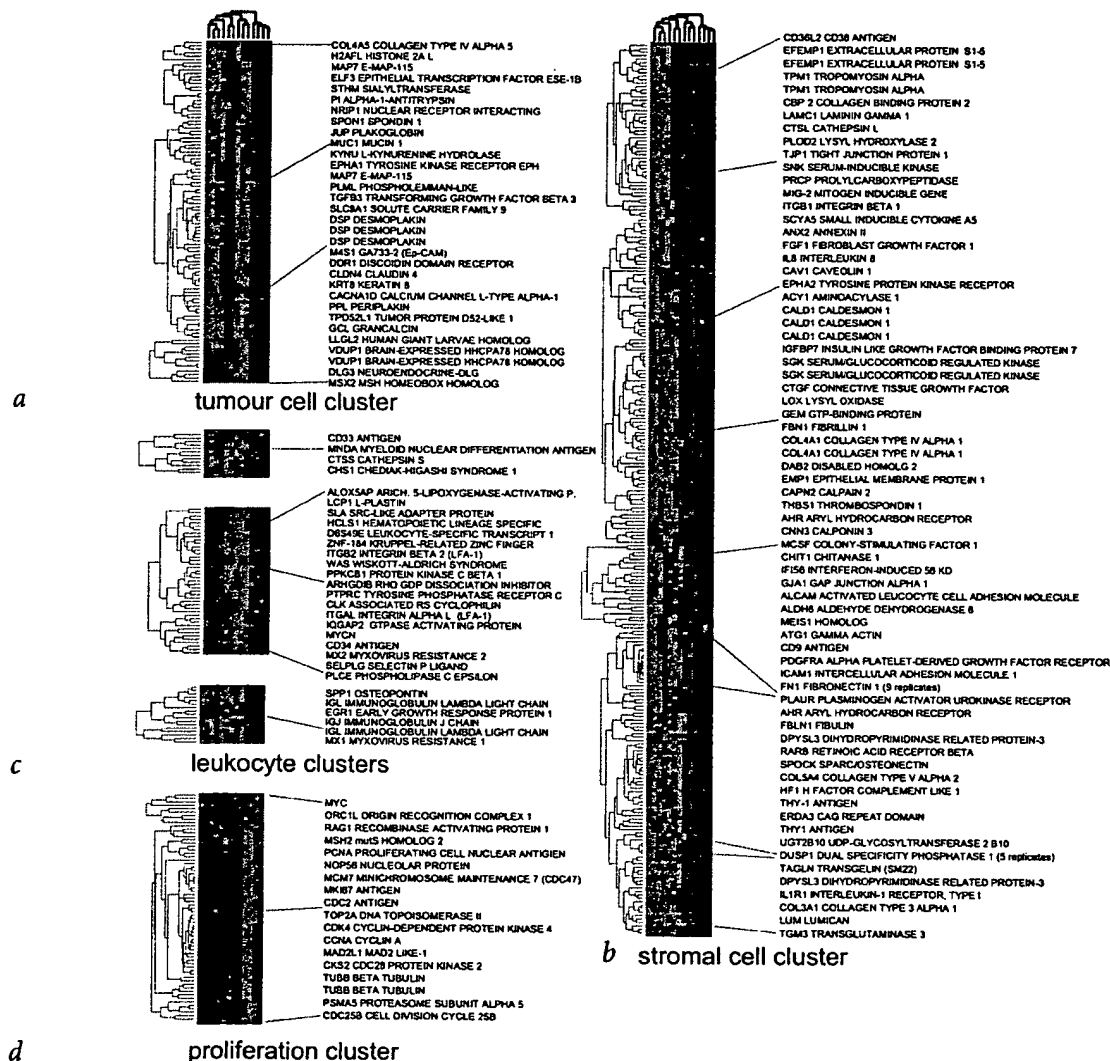
Fig. 5 Histologic features of breast cancer biopsies can be recognized and parsed based on gene expression patterns. Enlargements of the regions of the cluster diagram in Fig. 4 showing gene clusters enriched for genes expressed in different cell types in the breast cancer specimens, as distinguished by clustering with the cultured cell lines. **a**, A cluster including many genes characteristic of epithelial cells expressed in cell lines (T47D and MCF7) derived from breast cancer positive for the oestrogen receptor and tumours. **b**, Genes expressed in cell lines derived from breast cancer with stromal cell characteristics (Hs578T and BT549) and tumour specimens. Expression of these genes in the tumour samples may reflect the presence of myofibroblasts in the cancer specimen stroma. **c**, Genes expressed in leukocyte-derived cell lines, showing common leukocyte, and separate 'myeloid' and 'B-cell', gene clusters. **d**, Genes that were relatively highly expressed in all cell lines compared with the tumour specimens and normal breast. The higher expression of this set of genes involved in cell cycle transit in the cell lines is likely to reflect the higher proliferative rate of cells cultured in the presence of serum compared with the average proliferation rate of cells in the biopsied tissue.

We combined mRNA from the following cells in equal quantities to make the reference pool: HL-60 (acute myeloid leukaemia) and K562 (chronic myeloid leukaemia); NCI-H226 (non-small-cell-lung); COLO 205 (colon); SNB-19 (central nervous system); LOX-IMVI (melanoma); OVCAR-3 and OVCAR-4 (ovarian); CAKI-1 (renal); PC-3 (prostate); and MCF7 and Hs578T (breast). The criterion for selection of the cell lines in the reference are described in detail in the accompanying manuscript[12].

Doubling-time calculations. We calculated doubling times based on routine NCI60 cell line compound screening data; and they reflect the doubling times for cells inoculated into 96-well plates at the screening inoculation densities and grown in RPMI 1640 medium supplemented with 5% fetal bovine serum for 48 h. We measured cell populations using sulforhodamine B optical density measurement assay. The doubling time constant k was calculated using the equation: $N/No = e^{kt}$, where No is optical density for control (untreated) cells at time zero, N is optical density for control cells after 48-h incubation, and t is 48 h. The same equation was then used with the derived k to calculate the doubling time t by setting N/No = 2. For a given cell line, we obtained No and N values by averaging optical densities (N>6,000) obtained for each cell line for a year's screening. Data and experimental details are available (http://dtp.nci.nih.gov).

Preparation and hybridization of fluorescent labelled cDNA. For each comparative array hybridization, labelled cDNA was synthesized by reverse transcription from test cell mRNA in the presence of Cy5-dUTP, and from the reference mRNA with Cy3-dUTP, using the Superscript II reverse-transcription kit (Gibco-BRL). For each reverse transcription reaction, mRNA (2 µg) was mixed with an anchored oligo-dT (d-20T-d(AGC)) primer (4 µg) in a total volume of 15 µl, heated to 70 °C for 10 min and cooled on ice. To this sample, we added an unlabelled nucleotide pool (0.6 µl; 25 mM each dATP, dCTP, dGTP, and 15 mM dTTP), either Cy3 or Cy5 conjugated dUTP (3 µl; 1 mM; Amersham), 5×first-strand buffer (6 µl; 250 mM Tris-HCL, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 0.1 M DTT (3 µl) and 2 µl of Superscript II reverse transcriptase (200 µ/µl). After a 2-h incubation at 42 °C, the RNA was degraded by adding 1 N NaOH (1.5 µl) and incubating at 70 °C for 10 min. The mixture was neutralized by adding of 1 N HCL (1.5 µl), and the volume brought to 500 µl with TE (10 mM Tris, 1 mM EDTA). We added Cot1 human DNA (20 µg; Gibco-BRL), and purified the probe by centrifugation in a Centricon-30 micro-concentrator (Amicon). The two separate probes were combined, brought to a volume of 500 µl, and concentrated again to a volume of less than 7 µl. We added 10 µg/µl poly(A) RNA (1 µl; Sigma) and tRNA (10 µg/µl; Gibco-BRL) were added, and adjusted the volume to 9.5 µl with distilled water. For final probe preparation, 20×SSC (2.1 µl; 1.5 M NaCl, 150 mM NaCitrate, pH 8.0) and 10% SDS (0.35 µl) were added to a total final volume of 12 µl. The probes were denatured by heating for 2 min at 100 °C, incubated at 37 °C for 20–30 min, and placed on the array under a 22 mm×22 mm glass coverslip. We incubated slides overnight at 65 °C for 14–18 h in a custom slide chamber with humidity maintained by a small reservoir of 3×SSC. Arrays were washed by submersion and agitation for 2–5 min in 2×SSC with 0.1% SDS, followed by 1×SSC and then 0.1×SSC. The arrays were "spun dry" by centrifugation for 2 min in a slide-rack in a Beckman GS-6 tabletop centrifuge in Microplus carriers at 650 r.p.m. for 2 min.

Array quantitation and data processing. Following hybridization, arrays were scanned using a laser-scanning microscope (ref. 17; http://cmgm.stanford.edu/pbrown). Separate images were acquired for Cy3 and Cy5. We carried out data reduction with the program ScanAlyze (M.B.E., available

at http://rana.stanford.edu/software). Each spot was defined by manual positioning of a grid of circles over the array image. For each fluorescent image, the average pixel intensity within each circle was determined, and a local background was computed for each spot equal to the median pixel intensity in a square of 40 pixels in width and height centred on the spot centre, excluding all pixels within any defined spots. Net signal was determined by subtraction of this local background from the average intensity for each spot. Spots deemed unsuitable for accurate quantitation because of array artefacts were manually flagged and excluded from further analysis. Data files generated by ScanAlyze were entered into a custom database that maintains web-accessible files. Signal intensities between the two fluorescent images were normalized by applying a uniform scale factor to all intensities measured for the Cy5 channel. The normalization factor was chosen so that the mean log(Cy3/Cy5) for a subset of spots that achieved a minimum quality parameter (approximately 6,000 spots) was 0. This effectively defined the signal-intensity-weighted 'average' spot on each array to have a Cy3/Cy5 ratio of 1.0.

Cluster analysis. We extracted tables (rows of genes, columns of individual microarray hybridizations) of normalized fluorescence ratios from the database. Various selection criteria, discussed in relation to each data set, were applied to select subsets of genes from the 9,703 cDNA elements on the arrays. Before clustering and display, the logarithm of the measured fluorescence ratios for each gene were centred by subtracting the arithmetic mean of all ratios measured for that gene. The centring makes all subsequent analyses independent of the amount of each gene's mRNA in the reference pool.

We applied a hierarchical clustering algorithm separately to the cell lines and genes using the Pearson correlation coefficient as the measure of similarity and average linkage clustering[3,19–21]. The results of this process are two dendrograms (trees), one for the cell lines and one for the genes, in which very similar elements are connected by short branches, and longer branches join elements with diminishing degrees of similarity. For visual display the rows and columns in the initial data table were reordered to conform to the structures of the dendrograms obtained from the cluster analysis. Each cell in the cluster-ordered data table was replaced by a graded colour (pure red through black to pure green), representing the mean-adjusted ratio value in the cell. Gene labels in cluster diagrams are displayed here only for genes that were represented in the microarray by sequence-verified cDNAs. A complete software implementation of this process is available (http://rana.stanford.edu/software), as well as all clustering results (http://genome-www.stanford.edu/nci60).

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